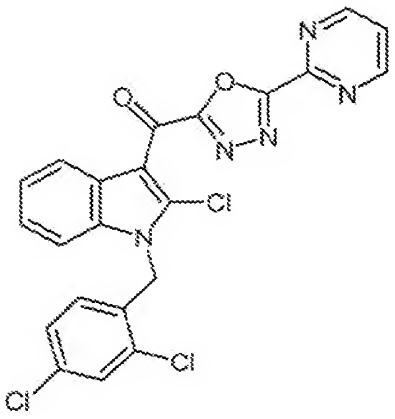
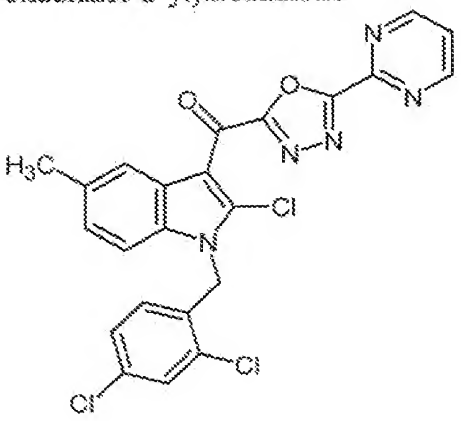
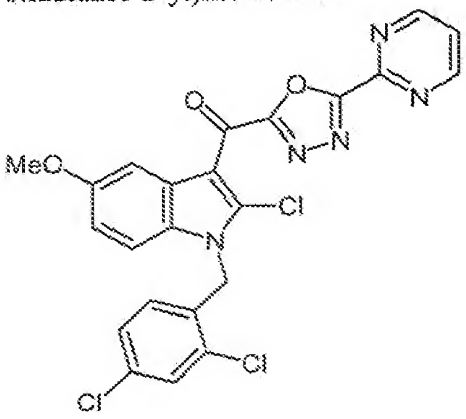


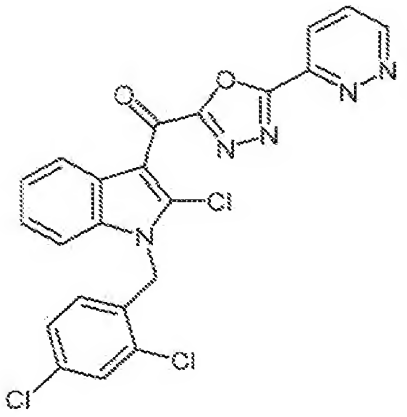
[2-chloro-1-(2,4-dichlorobenzyl)-5-methyl-1*H*-indol-3-yl](5-pyrimidin-2-yl-1,3,4-oxadiazol-2-yl)methanone



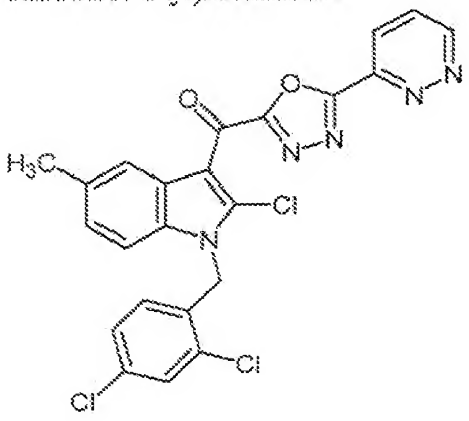
[2-chloro-1-(2,4-dichlorobenzyl)-5-methoxy-1*H*-indol-3-yl](5-pyrimidin-2-yl-1,3,4-oxadiazol-2-yl)methanone



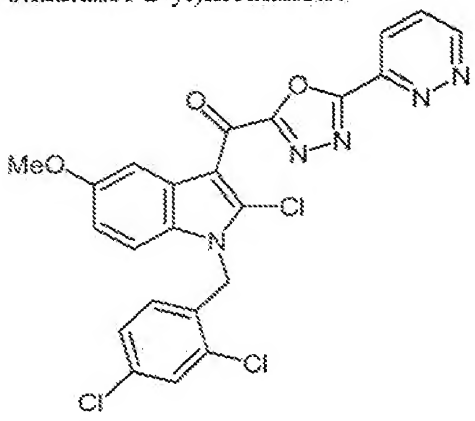
[2-chloro-1-(2,4-dichlorobenzyl)-1*H*-indol-3-yl](5-pyridazin-3-yl-1,3,4-oxadiazol-2-yl)methanone



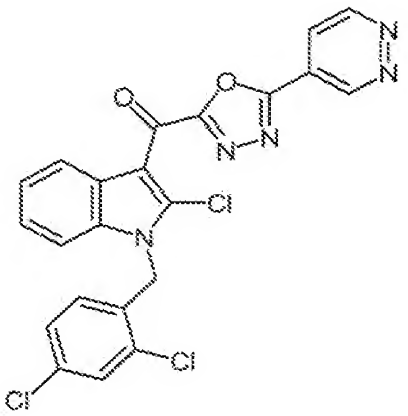
[2-chloro-1-(2,4-dichlorobenzyl)-5-methyl-1*H*-indol-3-yl](5-pyridazin-3-yl-1,3,4-oxadiazol-2-yl)methanone



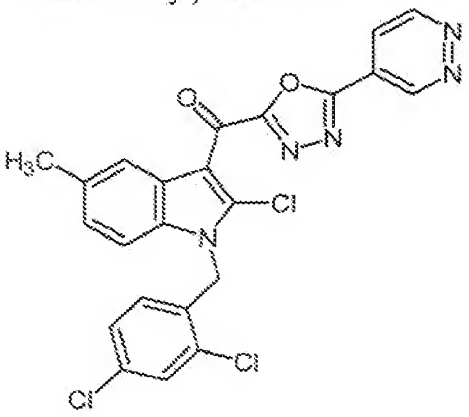
[2-chloro-1-(2,4-dichlorobenzyl)-5-methoxy-1*H*-indol-3-yl](5-pyridazin-3-yl-1,3,4-oxadiazol-2-yl)methanone



[2-chloro-1-(2,4-dichlorobenzyl)-1*H*-indol-3-yl](5-pyridazin-4-yl-1,3,4-oxadiazol-2-yl)methanone

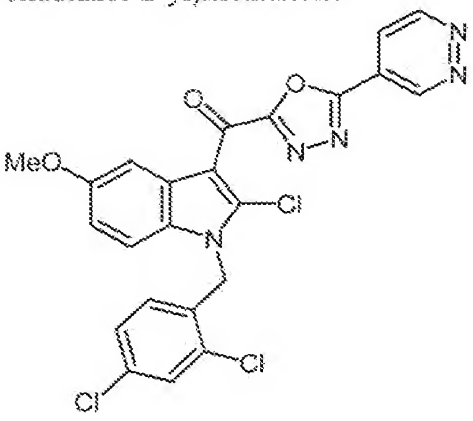


[2-chloro-1-(2,4-dichlorobenzyl)-5-methyl-1*H*-indol-3-yl](5-pyridazin-4-yl-1,3,4-oxadiazol-2-yl)methanone

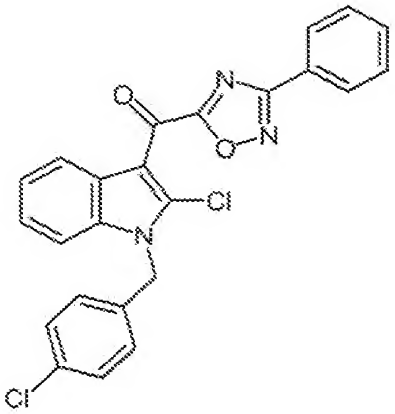


C, 55.39; H, 2.83; Cl, 21.33; N, 14.04; O, 6.42

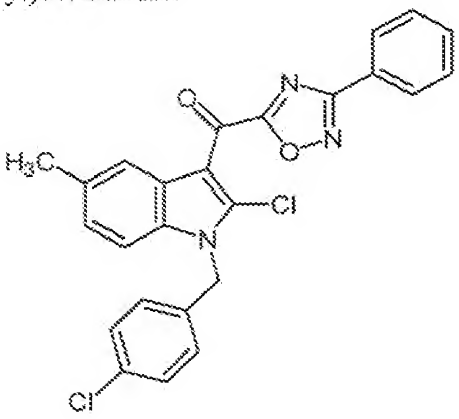
[2-chloro-1-(2,4-dichlorobenzyl)-5-methoxy-1*H*-indol-3-yl](5-pyridazin-4-yl-1,3,4-oxadiazol-2-yl)methanone



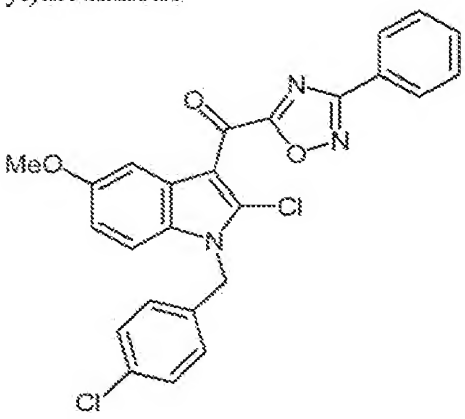
[2-chloro-1-(4-chlorobenzyl)-1*H*-indol-3-yl](3-phenyl-1,2,4-oxadiazol-5-yl)methanone



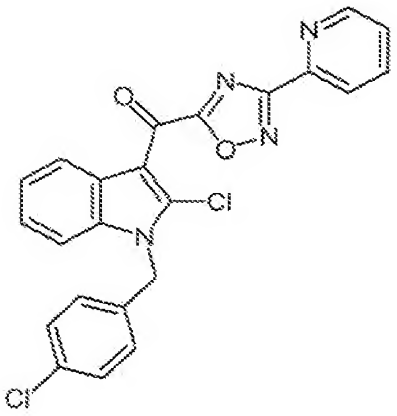
[2-chloro-1-(4-chlorobenzyl)-5-methyl-1*H*-indol-3-yl](3-phenyl-1,2,4-oxadiazol-5-yl)methanone



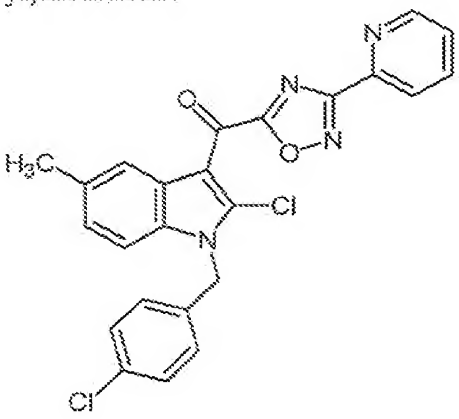
[2-chloro-1-(4-chlorobenzyl)-5-methoxy-1*H*-indol-3-yl](3-phenyl-1,2,4-oxadiazol-5-yl)methanone



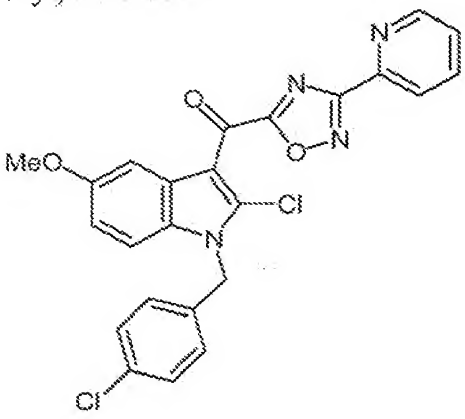
[2-chloro-1-(4-chlorobenzyl)-1*H*-indol-3-yl](3-pyridin-2-yl-1,2,4-oxadiazol-5-yl)methanone



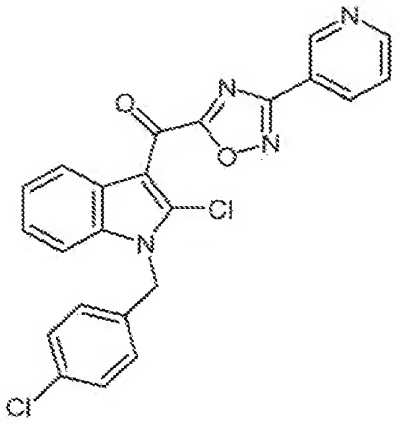
[2-chloro-1-(4-chlorobenzyl)-5-methyl-1*H*-indol-3-yl](3-pyridin-2-yl-1,2,4-oxadiazol-5-yl)methanone



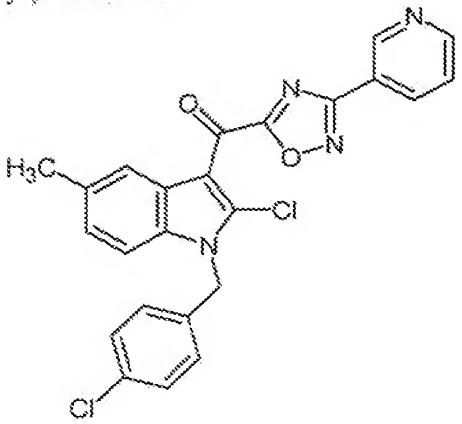
[2-chloro-1-(4-chlorobenzyl)-5-methoxy-1*H*-indol-3-yl](3-pyridin-2-yl-1,2,4-oxadiazol-5-yl)methanone



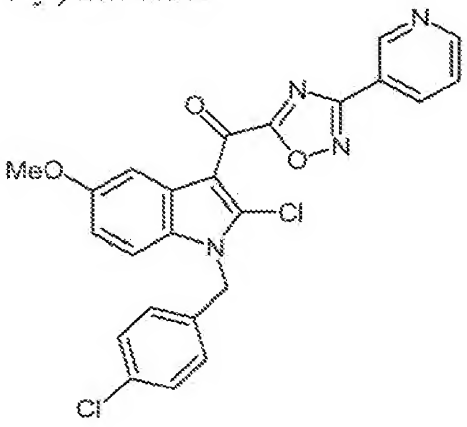
[2-chloro-1-(4-chlorobenzyl)-1*H*-indol-3-yl](3-pyridin-3-yl-1,2,4-oxadiazol-5-yl)methanone



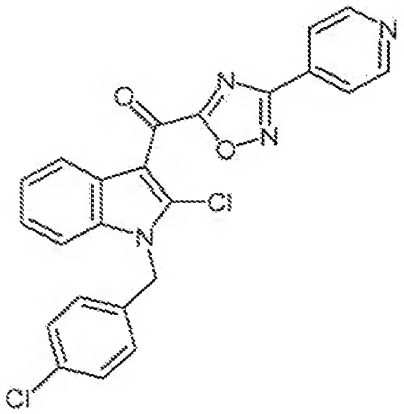
[2-chloro-1-(4-chlorobenzyl)-5-methyl-1*H*-indol-3-yl](3-pyridin-3-yl-1,2,4-oxadiazol-5-yl)methanone



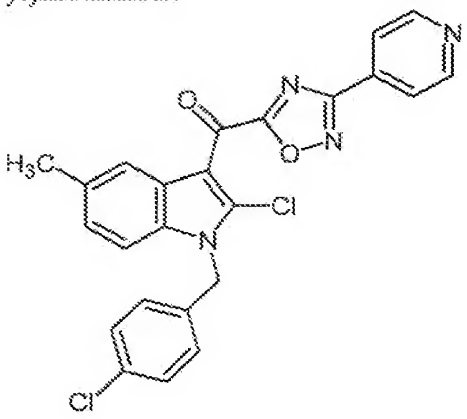
[2-chloro-1-(4-chlorobenzyl)-5-methoxy-1*H*-indol-3-yl](3-pyridin-3-yl-1,2,4-oxadiazol-5-yl)methanone



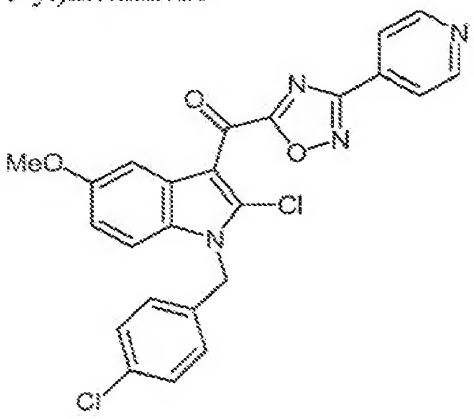
[2-chloro-1-(4-chlorobenzyl)-1*H*-indol-3-yl](3-pyridin-4-yl-1,2,4-oxadiazol-5-yl)methanone



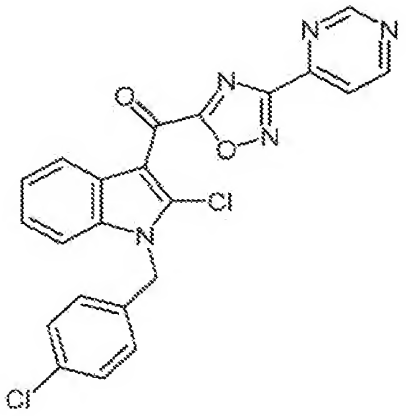
[2-chloro-1-(4-chlorobenzyl)-5-methyl-1*H*-indol-3-yl](3-pyridin-4-yl-1,2,4-oxadiazol-5-yl)methanone



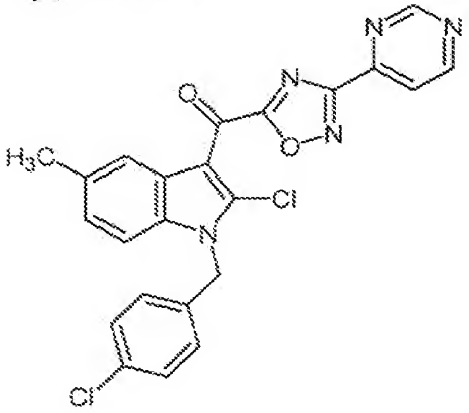
[2-chloro-1-(4-chlorobenzyl)-5-methoxy-1*H*-indol-3-yl](3-pyridin-4-yl-1,2,4-oxadiazol-5-yl)methanone



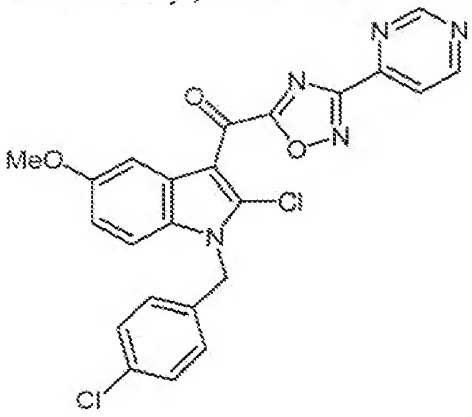
[2-chloro-1-(4-chlorobenzyl)-1*H*-indol-3-yl](3-pyrimidin-4-yl-1,2,4-oxadiazol-5-yl)methanone



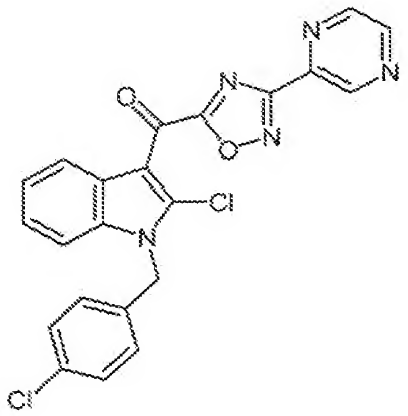
[2-chloro-1-(4-chlorobenzyl)-5-methyl-1*H*-indol-3-yl](3-pyrimidin-4-yl-1,2,4-oxadiazol-5-yl)methanone



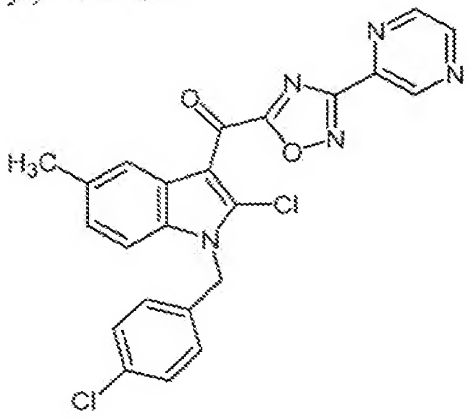
[2-chloro-1-(4-chlorobenzyl)-5-methoxy-1*H*-indol-3-yl](3-pyrimidin-4-yl-1,2,4-oxadiazol-5-yl)methanone



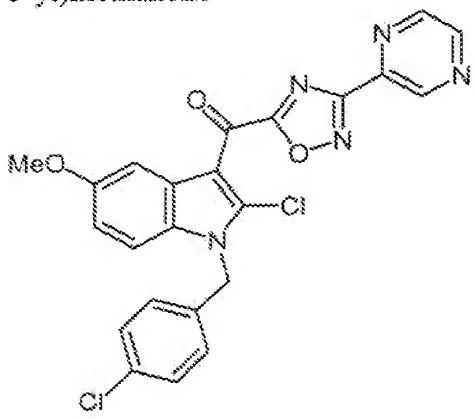
[2-chloro-1-(4-chlorobenzyl)-1*H*-indol-3-yl](3-pyrazin-2-yl-1,2,4-oxadiazol-5-yl)methanone



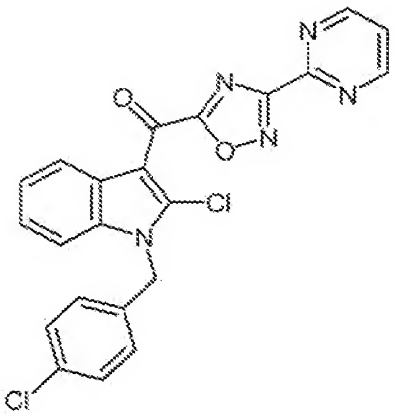
[2-chloro-1-(4-chlorobenzyl)-5-methyl-1*H*-indol-3-yl](3-pyrazin-2-yl-1,2,4-oxadiazol-5-yl)methanone



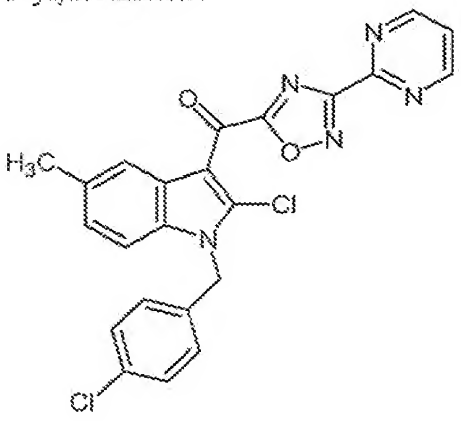
[2-chloro-1-(4-chlorobenzyl)-5-methoxy-1*H*-indol-3-yl](3-pyrazin-2-yl-1,2,4-oxadiazol-5-yl)methanone



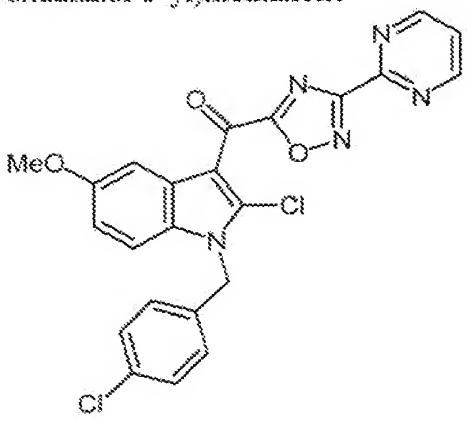
[2-chloro-1-(4-chlorobenzyl)-1*H*-indol-3-yl](3-pyrimidin-2-yl-1,2,4-oxadiazol-5-yl)methanone



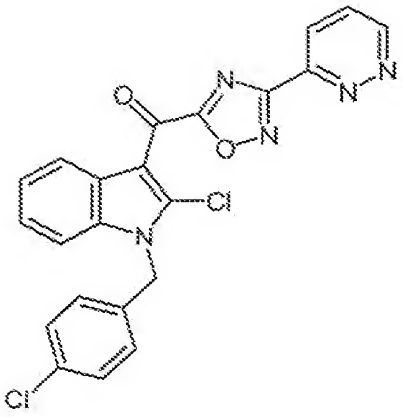
[2-chloro-1-(4-chlorobenzyl)-5-methyl-1*H*-indol-3-yl](3-pyrimidin-2-yl-1,2,4-oxadiazol-5-yl)methanone



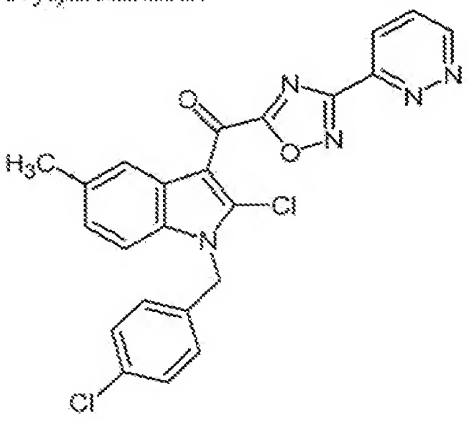
[2-chloro-1-(4-chlorobenzyl)-5-methoxy-1*H*-indol-3-yl](3-pyrimidin-2-yl-1,2,4-oxadiazol-5-yl)methanone



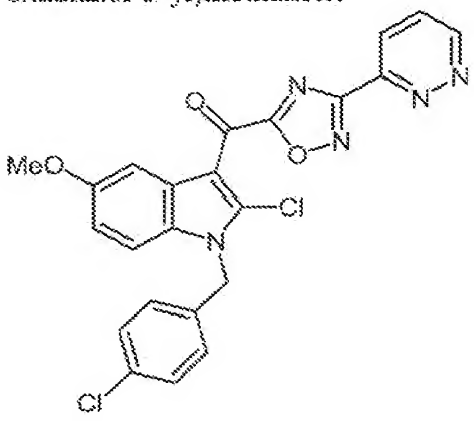
[2-chloro-1-(4-chlorobenzyl)-1*H*-indol-3-yl](3-pyridazin-3-yl-1,2,4-oxadiazol-5-yl)methanone



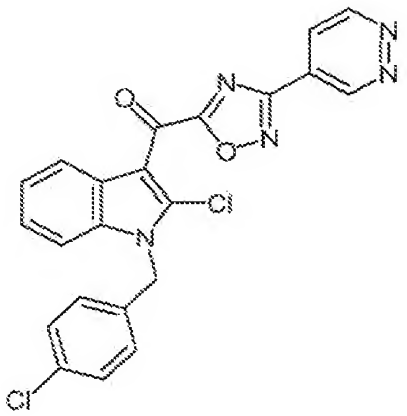
[2-chloro-1-(4-chlorobenzyl)-5-methyl-1*H*-indol-3-yl](3-pyridazin-3-yl-1,2,4-oxadiazol-5-yl)methanone



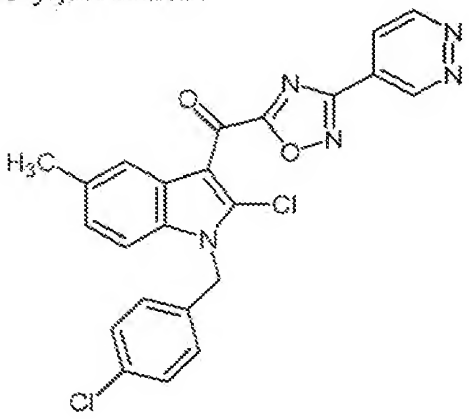
[2-chloro-1-(4-chlorobenzyl)-5-methoxy-1*H*-indol-3-yl](3-pyridazin-3-yl-1,2,4-oxadiazol-5-yl)methanone



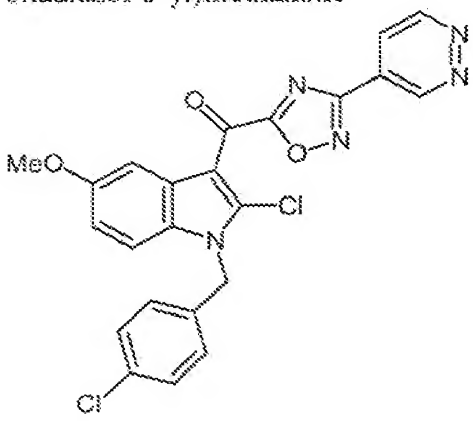
[2-chloro-1-(4-chlorobenzyl)-1*H*-indol-3-yl](3-pyridazin-4-yl-1,2,4-oxadiazol-5-yl)methanone



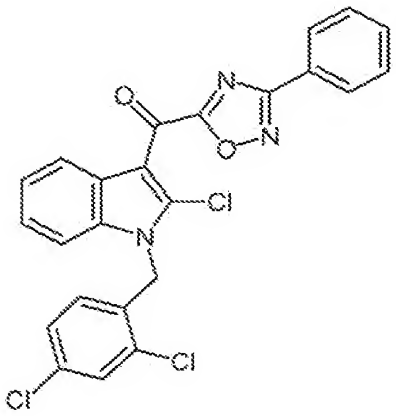
[2-chloro-1-(4-chlorobenzyl)-5-methyl-1*H*-indol-3-yl](3-pyridazin-4-yl-1,2,4-oxadiazol-5-yl)methanone



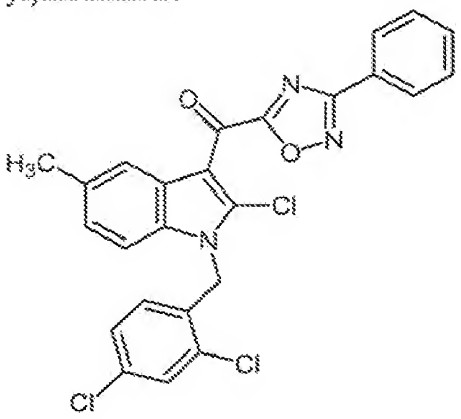
[2-chloro-1-(4-chlorobenzyl)-5-methoxy-1*H*-indol-3-yl](3-pyridazin-4-yl-1,2,4-oxadiazol-5-yl)methanone



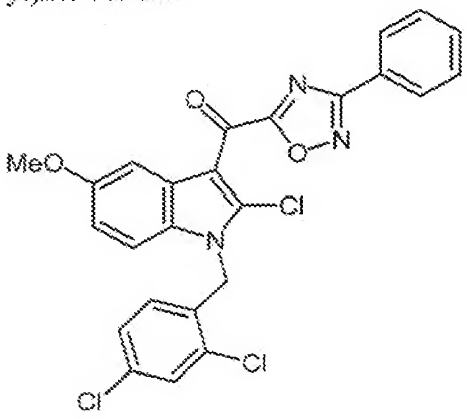
[2-chloro-1-(2,4-dichlorobenzyl)-1*H*-indol-3-yl](3-phenyl-1,2,4-oxadiazol-5-yl)methanone



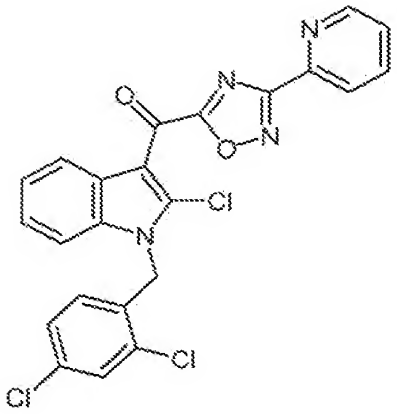
[2-chloro-1-(2,4-dichlorobenzyl)-5-methyl-1*H*-indol-3-yl](3-phenyl-1,2,4-oxadiazol-5-yl)methanone



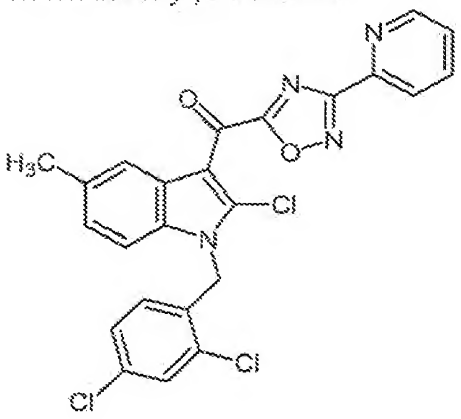
[2-chloro-1-(2,4-dichlorobenzyl)-5-methoxy-1*H*-indol-3-yl](3-phenyl-1,2,4-oxadiazol-5-yl)methanone



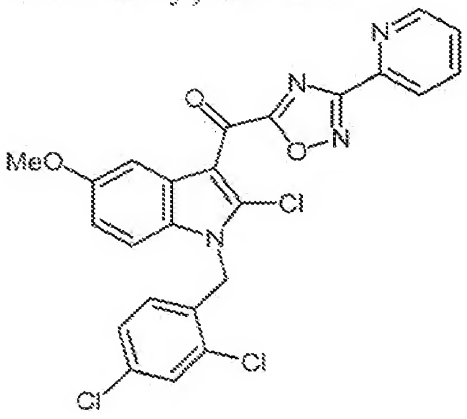
[2-chloro-1-(2,4-dichlorobenzyl)-1*H*-indol-3-yl](3-pyridin-2-yl-1,2,4-oxadiazol-5-yl)methanone



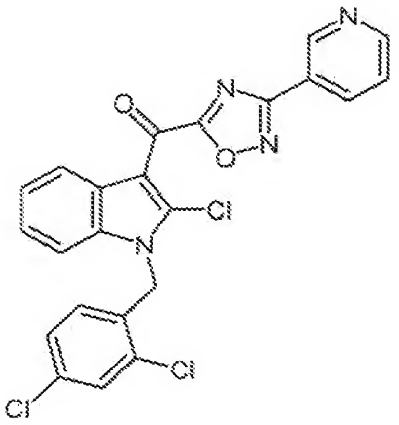
[2-chloro-1-(2,4-dichlorobenzyl)-5-methyl-1*H*-indol-3-yl](3-pyridin-2-yl-1,2,4-oxadiazol-5-yl)methanone



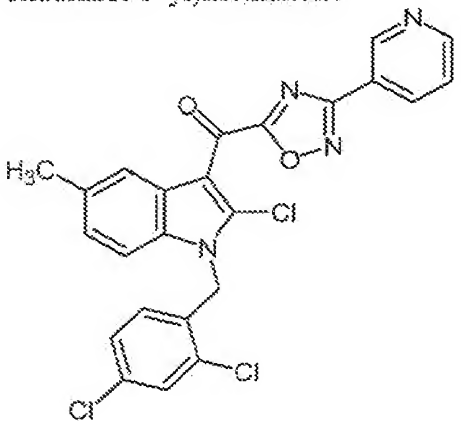
[2-chloro-1-(2,4-dichlorobenzyl)-5-methoxy-1*H*-indol-3-yl](3-pyridin-2-yl-1,2,4-oxadiazol-5-yl)methanone



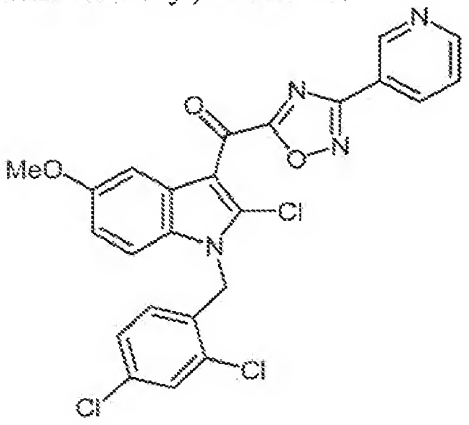
[2-chloro-1-(2,4-dichlorobenzyl)-1*H*-indol-3-yl](3-pyridin-3-yl-1,2,4-oxadiazol-5-yl)methanone



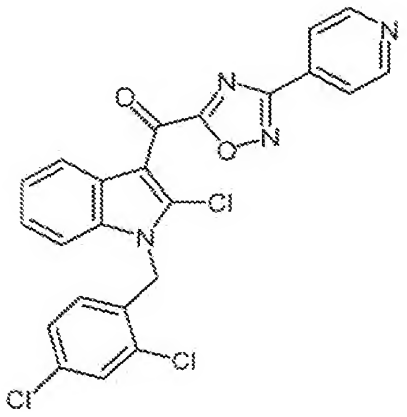
[2-chloro-1-(2,4-dichlorobenzyl)-5-methyl-1*H*-indol-3-yl](3-pyridin-3-yl-1,2,4-oxadiazol-5-yl)methanone



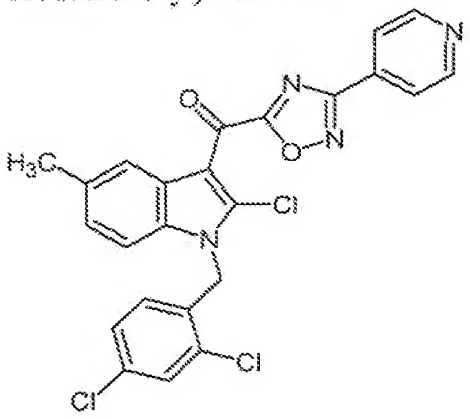
[2-chloro-1-(2,4-dichlorobenzyl)-5-methoxy-1*H*-indol-3-yl](3-pyridin-3-yl-1,2,4-oxadiazol-5-yl)methanone



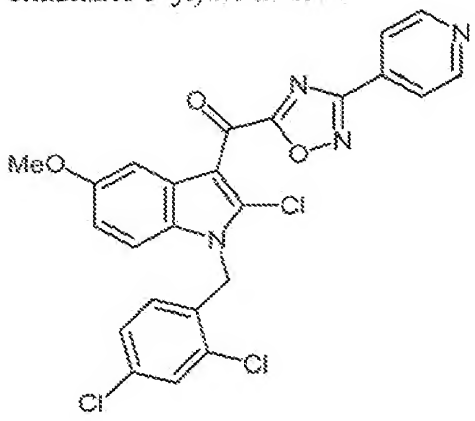
[2-chloro-1-(2,4-dichlorobenzyl)-1*H*-indol-3-yl](3-pyridin-4-yl-1,2,4-oxadiazol-5-yl)methanone



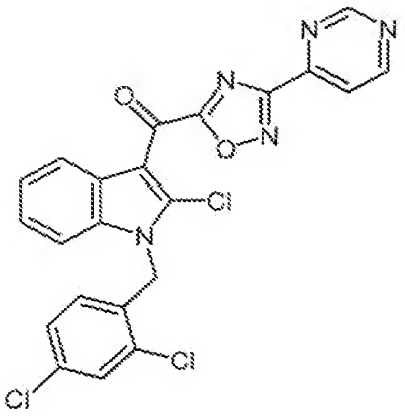
[2-chloro-1-(2,4-dichlorobenzyl)-5-methyl-1*H*-indol-3-yl](3-pyridin-4-yl-1,2,4-oxadiazol-5-yl)methanone



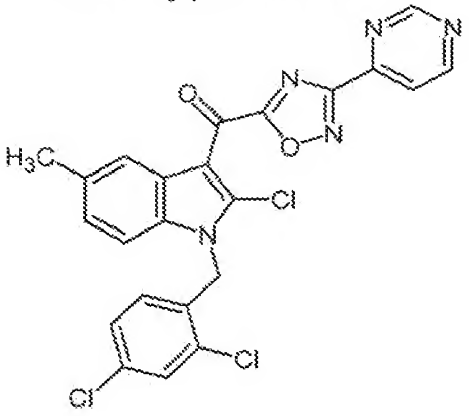
[2-chloro-1-(2,4-dichlorobenzyl)-5-methoxy-1*H*-indol-3-yl](3-pyridin-4-yl-1,2,4-oxadiazol-5-yl)methanone



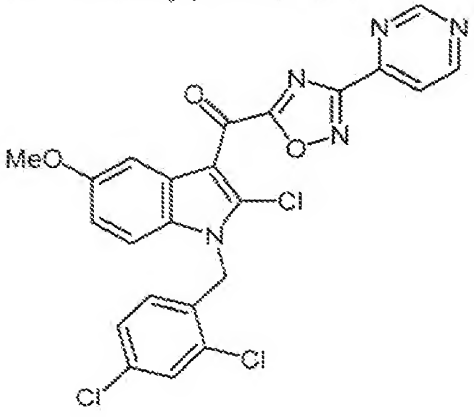
[2-chloro-1-(2,4-dichlorobenzyl)-1*H*-indol-3-yl](3-pyrimidin-4-yl-1,2,4-oxadiazol-5-yl)methanone



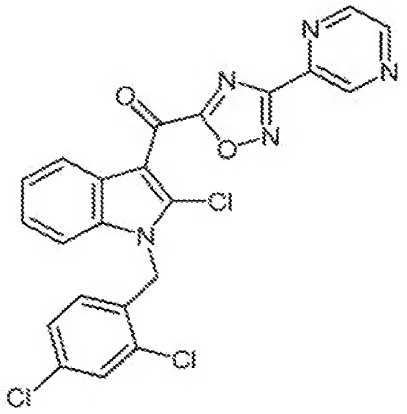
[2-chloro-1-(2,4-dichlorobenzyl)-5-methyl-1*H*-indol-3-yl](3-pyrimidin-4-yl-1,2,4-oxadiazol-5-yl)methanone



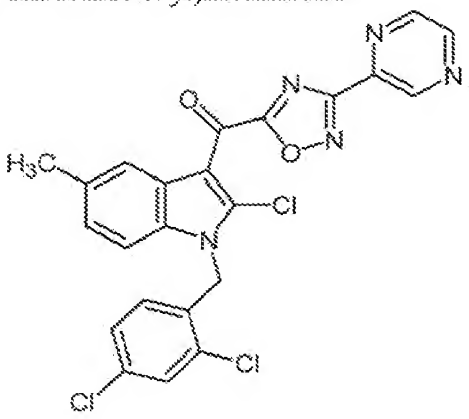
[2-chloro-1-(2,4-dichlorobenzyl)-5-methoxy-1*H*-indol-3-yl](3-pyrimidin-4-yl-1,2,4-oxadiazol-5-yl)methanone



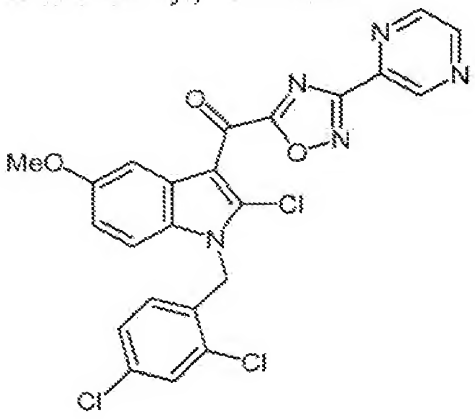
[2-chloro-1-(2,4-dichlorobenzyl)-1*H*-indol-3-yl](3-pyrazin-2-yl-1,2,4-oxadiazol-5-yl)methanone



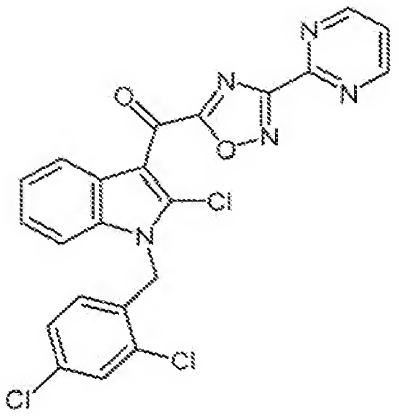
[2-chloro-1-(2,4-dichlorobenzyl)-5-methyl-1*H*-indol-3-yl](3-pyrazin-2-yl-1,2,4-oxadiazol-5-yl)methanone



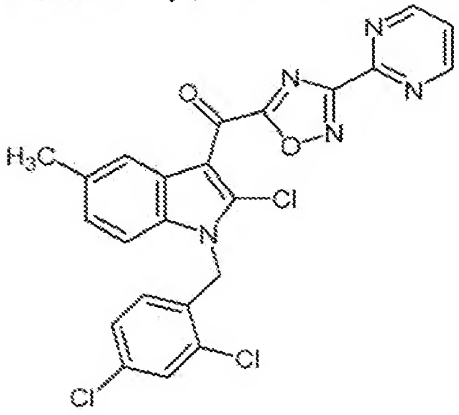
[2-chloro-1-(2,4-dichlorobenzyl)-5-methoxy-1*H*-indol-3-yl](3-pyrazin-2-yl-1,2,4-oxadiazol-5-yl)methanone



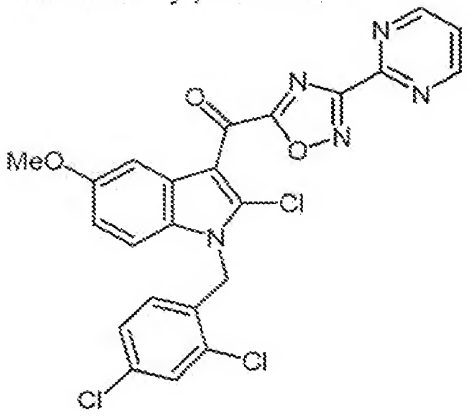
[2-chloro-1-(2,4-dichlorobenzyl)-1*H*-indol-3-yl](3-pyrimidin-2-yl-1,2,4-oxadiazol-5-yl)methanone



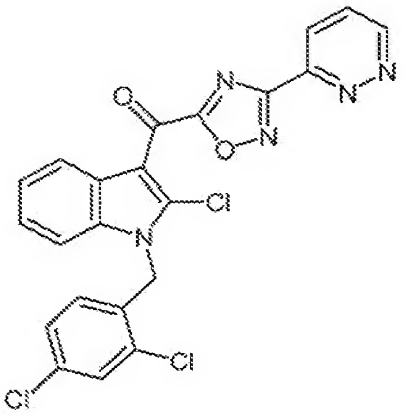
[2-chloro-1-(2,4-dichlorobenzyl)-5-methyl-1*H*-indol-3-yl](3-pyrimidin-2-yl-1,2,4-oxadiazol-5-yl)methanone



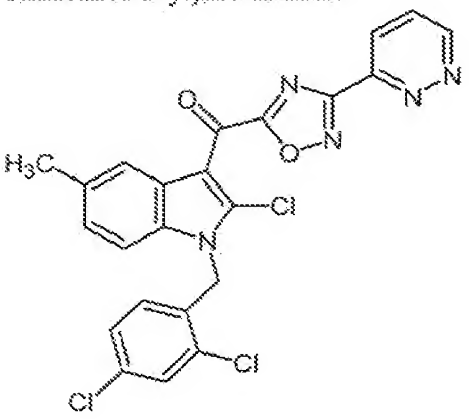
[2-chloro-1-(2,4-dichlorobenzyl)-5-methoxy-1*H*-indol-3-yl](3-pyrimidin-2-yl-1,2,4-oxadiazol-5-yl)methanone



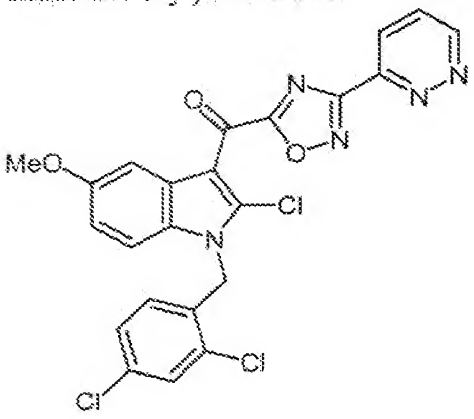
[2-chloro-1-(2,4-dichlorobenzyl)-1*H*-indol-3-yl](3-pyridazin-3-yl-1,2,4-oxadiazol-5-yl)methanone



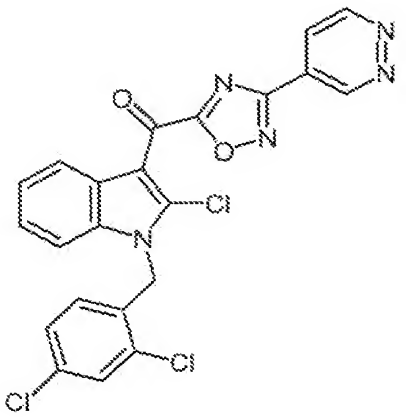
[2-chloro-1-(2,4-dichlorobenzyl)-5-methyl-1*H*-indol-3-yl](3-pyridazin-3-yl-1,2,4-oxadiazol-5-yl)methanone



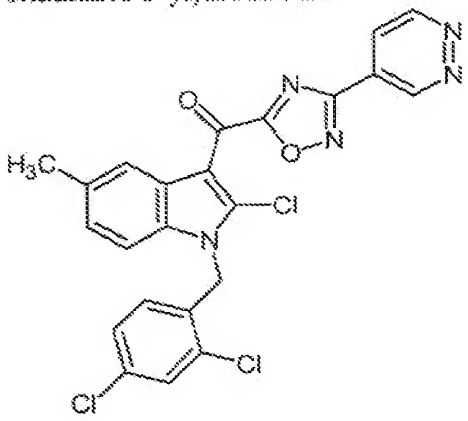
[2-chloro-1-(2,4-dichlorobenzyl)-5-methoxy-1*H*-indol-3-yl](3-pyridazin-3-yl-1,2,4-oxadiazol-5-yl)methanone



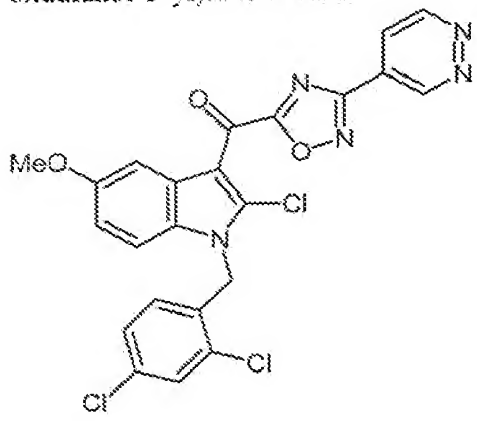
[2-chloro-1-(2,4-dichlorobenzyl)-1*H*-indol-3-yl](3-pyridazin-4-yl-1,2,4-oxadiazol-5-yl)methanone



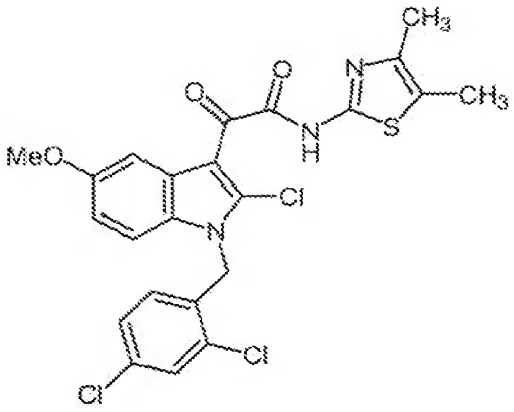
[2-chloro-1-(2,4-dichlorobenzyl)-5-methyl-1*H*-indol-3-yl](3-pyridazin-4-yl-1,2,4-oxadiazol-5-yl)methanone



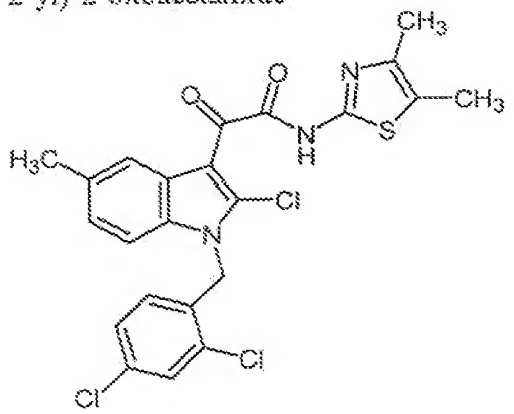
[2-chloro-1-(2,4-dichlorobenzyl)-5-methoxy-1*H*-indol-3-yl](3-pyridazin-4-yl-1,2,4-oxadiazol-5-yl)methanone



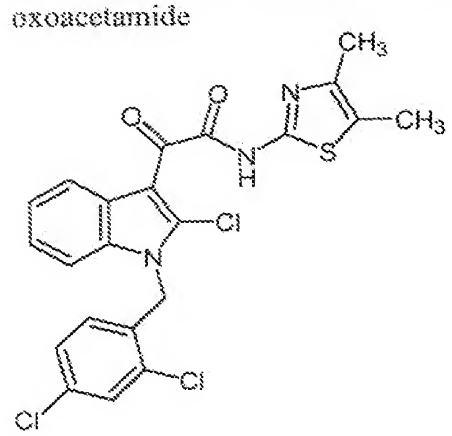
2-[2-chloro-1-(2,4-dichlorobenzyl)-5-methoxy-1*H*-indol-3-yl]-*N*-(4,5-dimethyl-1,3-thiazol-2-yl)-2-oxoacetamide



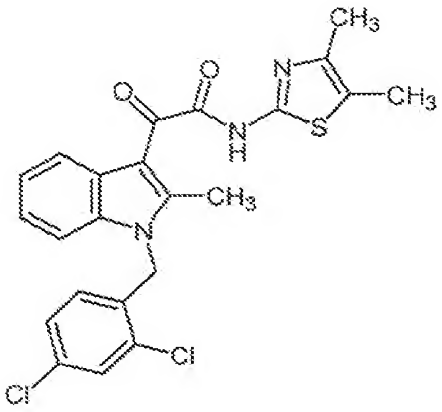
2-[2-chloro-1-(2,4-dichlorobenzyl)-5-methyl-1*H*-indol-3-yl]-*N*-(4,5-dimethyl-1,3-thiazol-2-yl)-2-oxoacetamide



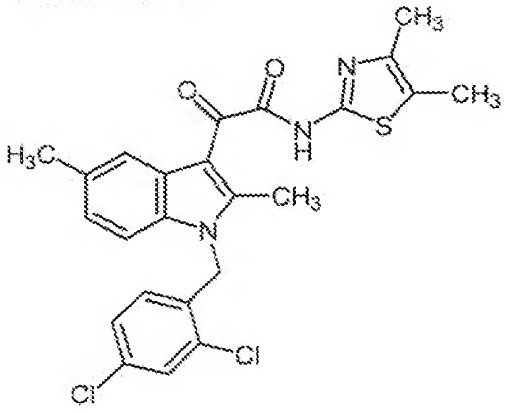
2-[2-chloro-1-(2,4-dichlorobenzyl)-1*H*-indol-3-yl]-*N*-(4,5-dimethyl-1,3-thiazol-2-yl)-2-oxoacetamide



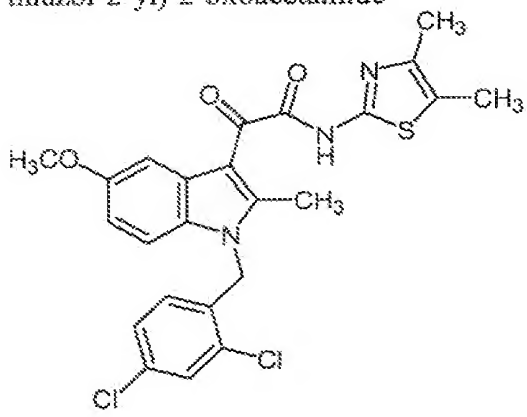
2-[1-(2,4-dichlorobenzyl)-2-methyl-1*H*-indol-3-yl]-*N*-(4,5-dimethyl-1,3-thiazol-2-yl)-2-oxoacetamide



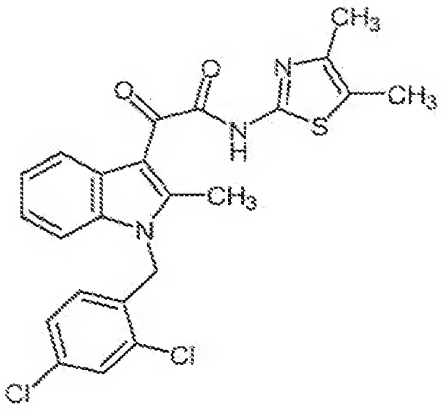
2-[1-(2,4-dichlorobenzyl)-2,5-dimethyl-1*H*-indol-3-yl]-*N*-(4,5-dimethyl-1,3-thiazol-2-yl)-2-oxoacetamide



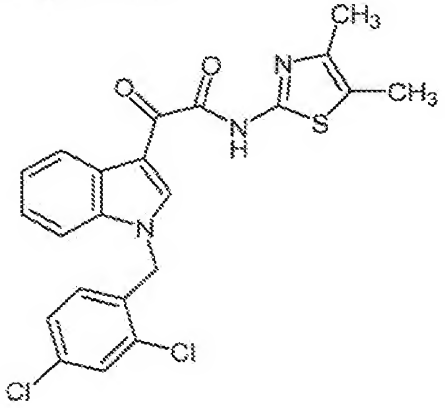
2-[1-(2,4-dichlorobenzyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]-*N*-(4,5-dimethyl-1,3-thiazol-2-yl)-2-oxoacetamide



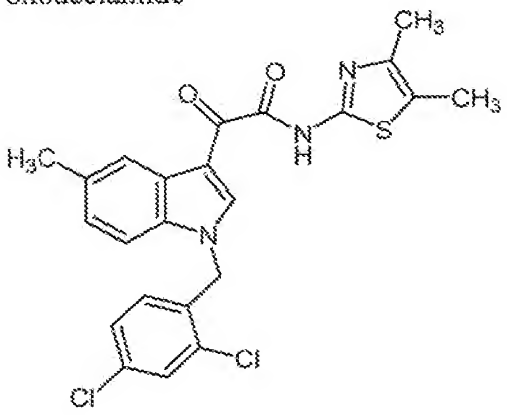
2-[1-(2,4-dichlorobenzyl)-2-methyl-1*H*-indol-3-yl]-*N*-(4,5-dimethyl-1,3-thiazol-2-yl)-2-oxoacetamide



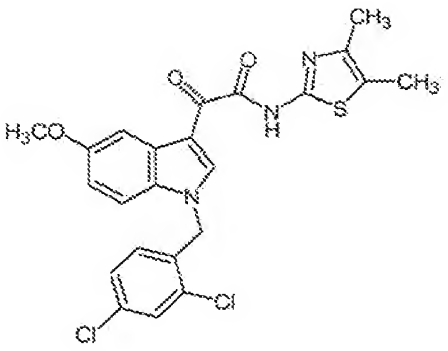
2-[1-(2,4-dichlorobenzyl)-1*H*-indol-3-yl]-*N*-(4,5-dimethyl-1,3-thiazol-2-yl)-2-oxoacetamide



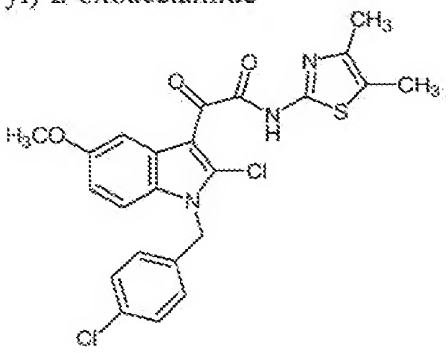
2-[1-(2,4-dichlorobenzyl)-5-methyl-1*H*-indol-3-yl]-*N*-(4,5-dimethyl-1,3-thiazol-2-yl)-2-oxoacetamide



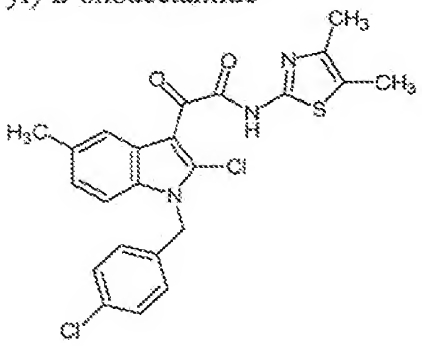
2-[1-(2,4-dichlorobenzyl)-5-methoxy-1*H*-indol-3-yl]-*N*-(4,5-dimethyl-1,3-thiazol-2-yl)-2-oxoacetamide



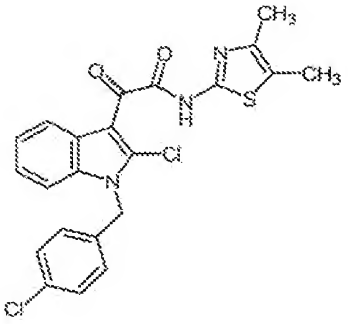
2-[2-chloro-1-(4-chlorobenzyl)-5-methoxy-1*H*-indol-3-yl]-*N*-(4,5-dimethyl-1,3-thiazol-2-yl)-2-oxoacetamide



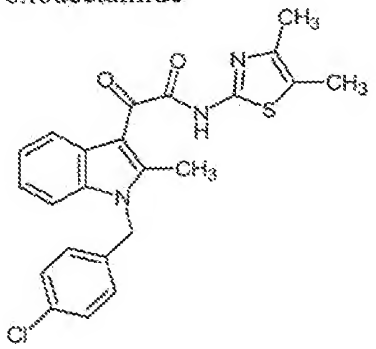
2-[2-chloro-1-(4-chlorobenzyl)-5-methyl-1*H*-indol-3-yl]-*N*-(4,5-dimethyl-1,3-thiazol-2-yl)-2-oxoacetamide



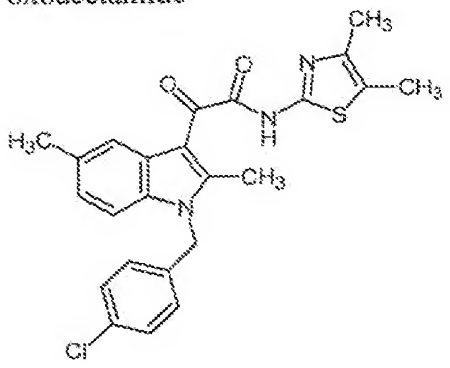
2-[2-chloro-1-(4-chlorobenzyl)-1*H*-indol-3-yl]-*N*-(4,5-dimethyl-1,3-thiazol-2-yl)-2-oxoacetamide



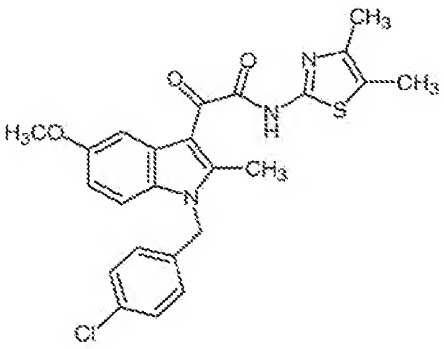
2-[1-(4-chlorobenzyl)-2-methyl-1*H*-indol-3-yl]-*N*-(4,5-dimethyl-1,3-thiazol-2-yl)-2-oxoacetamide



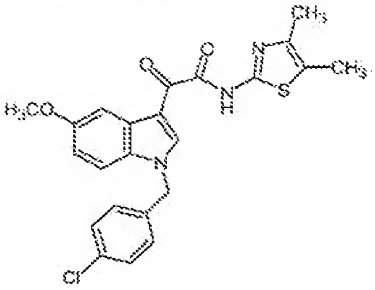
2-[1-(4-chlorobenzyl)-2,5-dimethyl-1*H*-indol-3-yl]-*N*-(4,5-dimethyl-1,3-thiazol-2-yl)-2-oxoacetamide



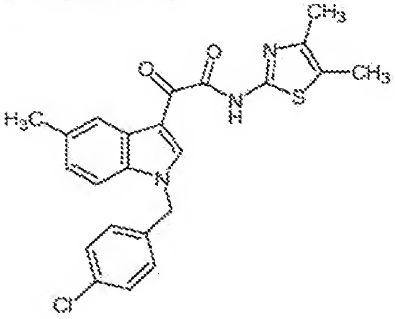
2-[1-(4-chlorobenzyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]-*N*-(4,5-dimethyl-1,3-thiazol-2-yl)-2-oxoacetamide



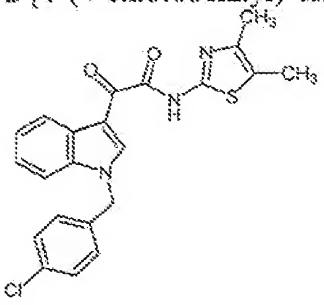
2-[1-(4-chlorobenzyl)-5-methoxy-1*H*-indol-3-yl]-*N*-(4,5-dimethyl-1,3-thiazol-2-yl)-2-oxoacetamide



2-[1-(4-chlorobenzyl)-5-methyl-1*H*-indol-3-yl]-*N*-(4,5-dimethyl-1,3-thiazol-2-yl)-2-oxoacetamide

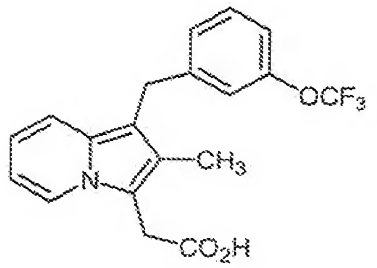


2-[1-(4-chlorobenzyl)-1*H*-indol-3-yl]-*N*-(4,5-dimethyl-1,3-thiazol-2-yl)-2-oxoacetamide

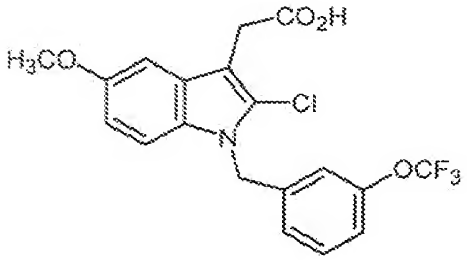


CRTH2 inhibitors

[2-methyl-1-(3-trifluoromethoxylbenzyl)indolizin-3-yl]acetic acid



[2-chloro-5-methoxy-1-(3-trifluoromethoxylbenzyl)-1H-indol-3-yl]acetic acid



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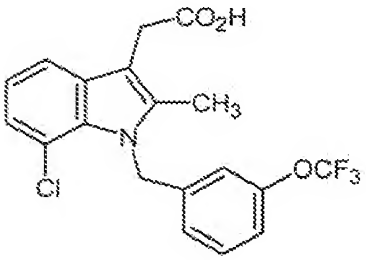

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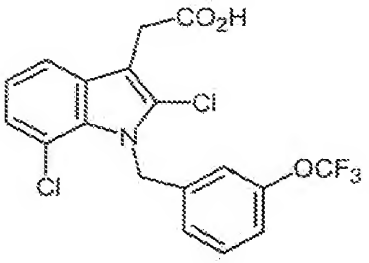

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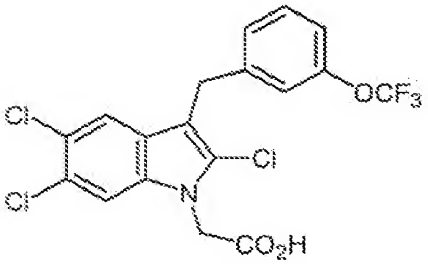

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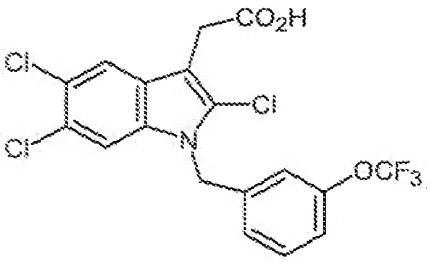

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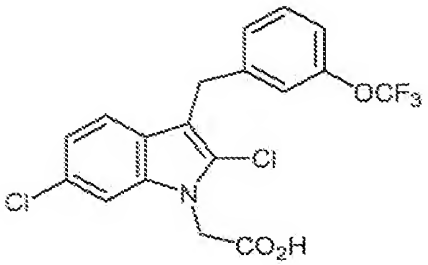

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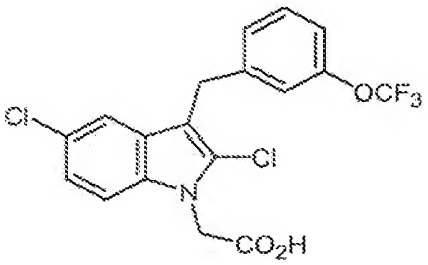
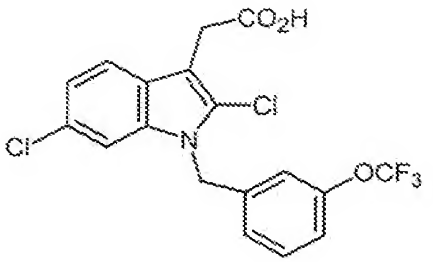
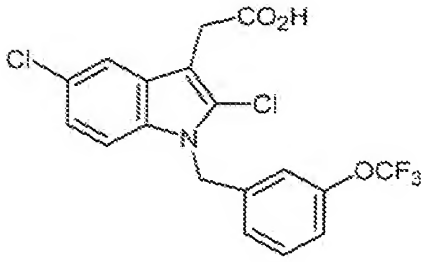


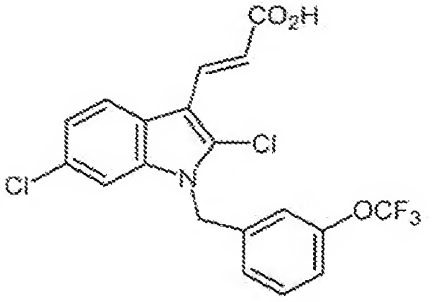
[2,6-dichloro-1-(3-trifluoromethoxylbenzy)-1H-indol-3-yl]acetic acid



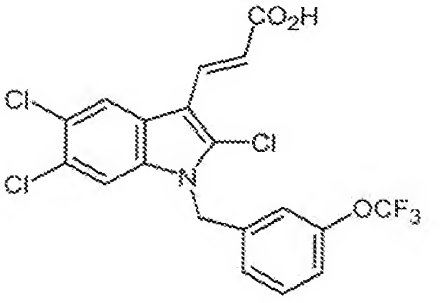
[2,5-dichloro-1-(3-trifluoromethoxylbenzy)-1H-indol-3-yl]acetic acid



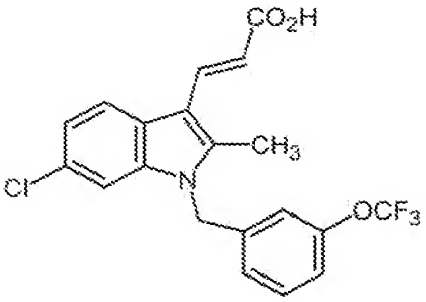
(2E)-3-[2,6-dichloro-1-(3-trifluoromethoxylbenzy)-1H-indol-3-yl]acrylic acid



(2E)-3-[2,5,6-trichloro-1-(3-trifluoromethoxylbenzy)-1H-indol-3-yl]acrylic acid



(2E)-3-[6-chloro-2-methyl-1-(3-trifluoromethoxylbenzy)-1H-indol-3-yl]acrylic acid



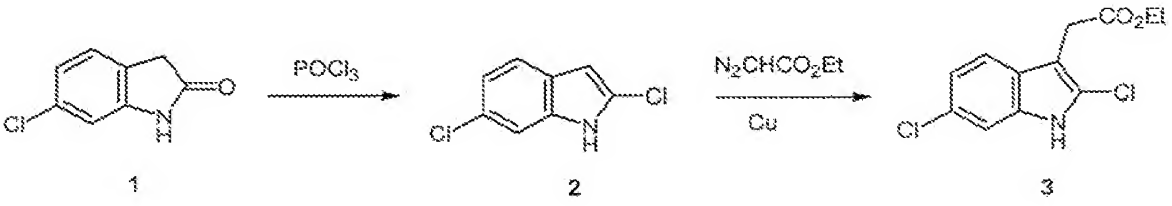
Synthetic Methods

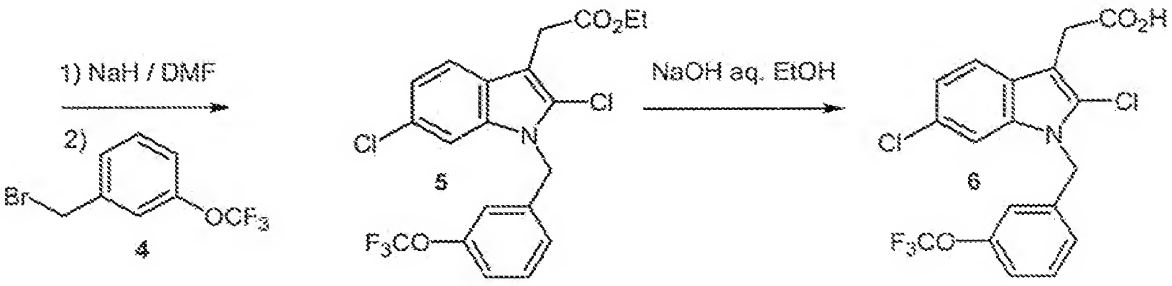
General synthetic methods for the preparation of compounds described herein include:

Section 1.

General synthetic methods for the preparation of compounds described above:

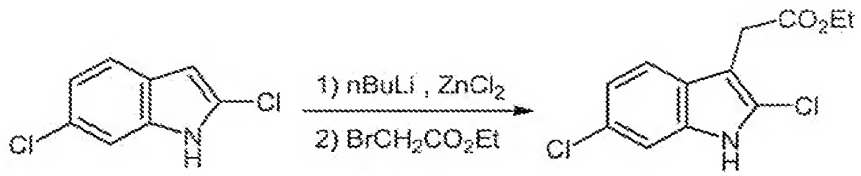
Scheme 1.





Jackson, R. W.; Manske, R. H. *Can. J. Research* **1935**, *13*, 170-174. Wenkert, E.; Alonso, M. E.; Gottlieb, H. E.; Sanchez, E. L.; Pellicciari, R.; Cogolli, P. *J. Org. Chem.* **1977**, *42*, 3945-3949.

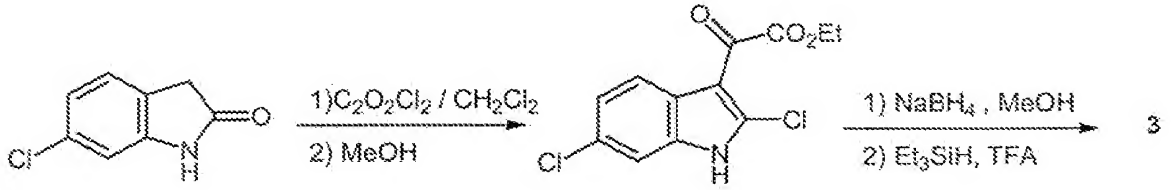
Scheme 2



2 3

Dillard, R. D.; Bach, N. J.; Draheim, S. E.; Berry, D. R.; Carlson, D. G.; Chirgadze, N. Y.; Clawson, D. K.; Hartley, L. W.; Johnson, L. M.; Jones, N. D.; McKinney, E. R.; Mihelich, E. D.; Olkowski, J. L.; Schevitz, R. W.; Smith, A. C.; Snyder, D. W.; Sommers, C. D.; Wery, J. P. *J. Med. Chem.* **1996**, *39*, 5119-5136.

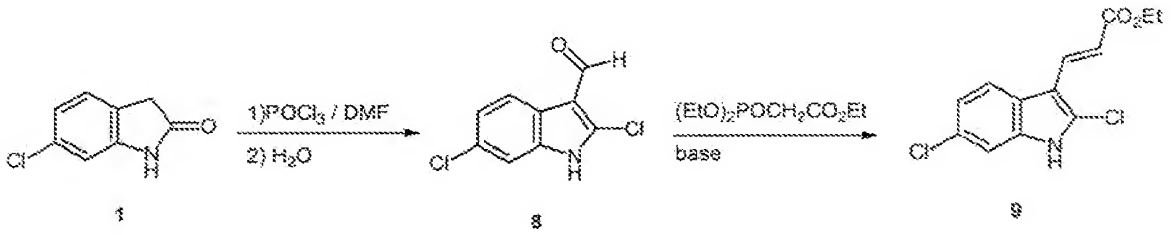
Scheme 3.



1 7

Collot, V.; Schmitt, M.; Marwah, P.; Bourguignon, J. J. *Heterocycles* **1999**, *51*, 2823-2847.

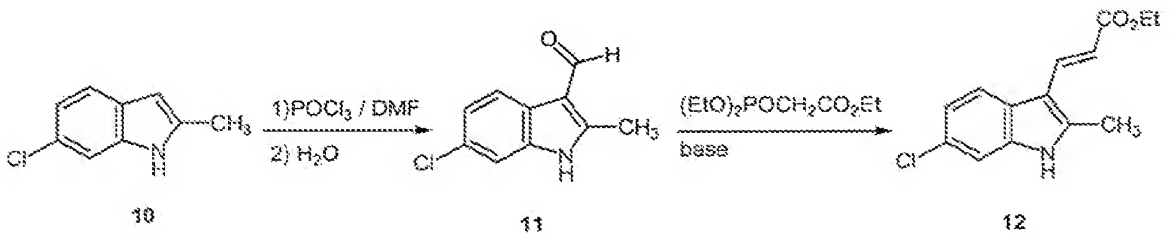
Scheme 4.



1 8 9

Padras, M. S. C.; Jha, M. *J. Org. Chem.* **2005**, *70*, 1828-1834

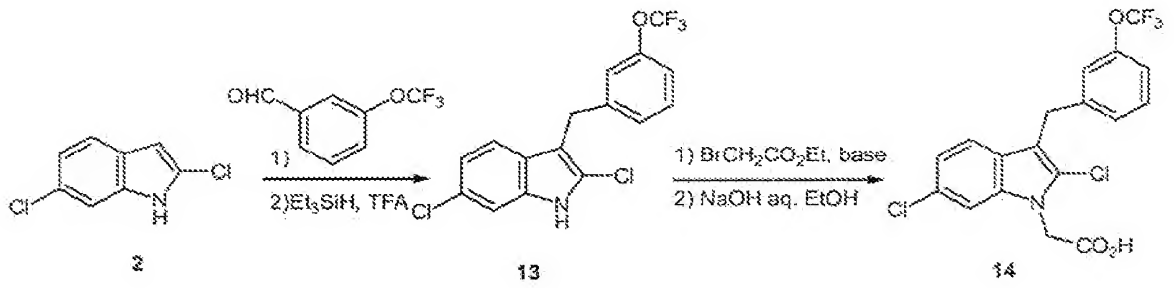
Scheme 5.



10 11 12

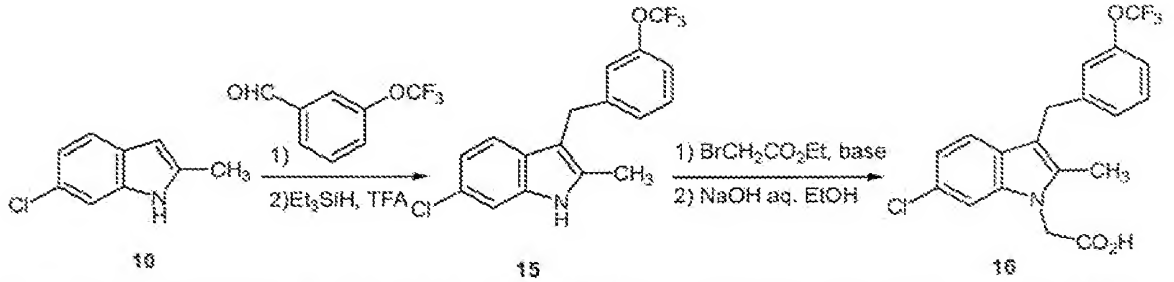
Mahboobi, S.; Eibler, E.; Koller, M.; Kumar, S.; Popp, A. *J. Org. Chem.* **1999**, *64*, 4697-4704.

Scheme 6.



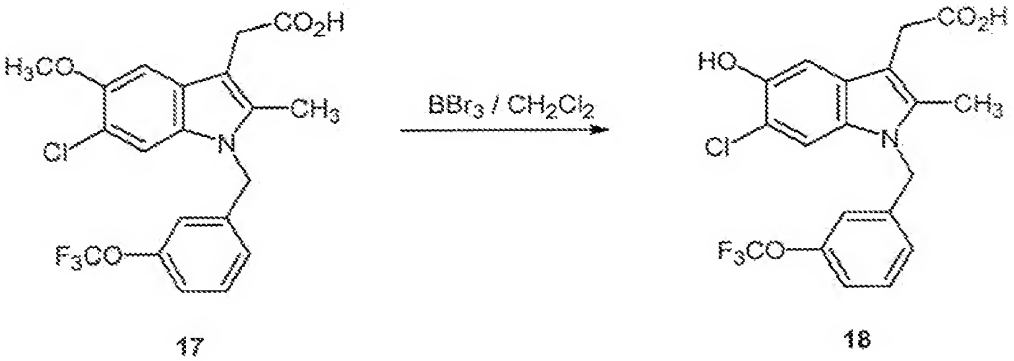
Appleton, J. E.; Dack, K. N.; Green, A. D.; Steele, J. *Tetrahedron Lett.* **1993**, *34*, 1529-1532.

Scheme 7.



Appleton, J. E.; Dack, K. N.; Green, A. D.; Steele, J. *Tetrahedron Lett.* **1993**, *34*, 1529-1532.

Scheme 8.

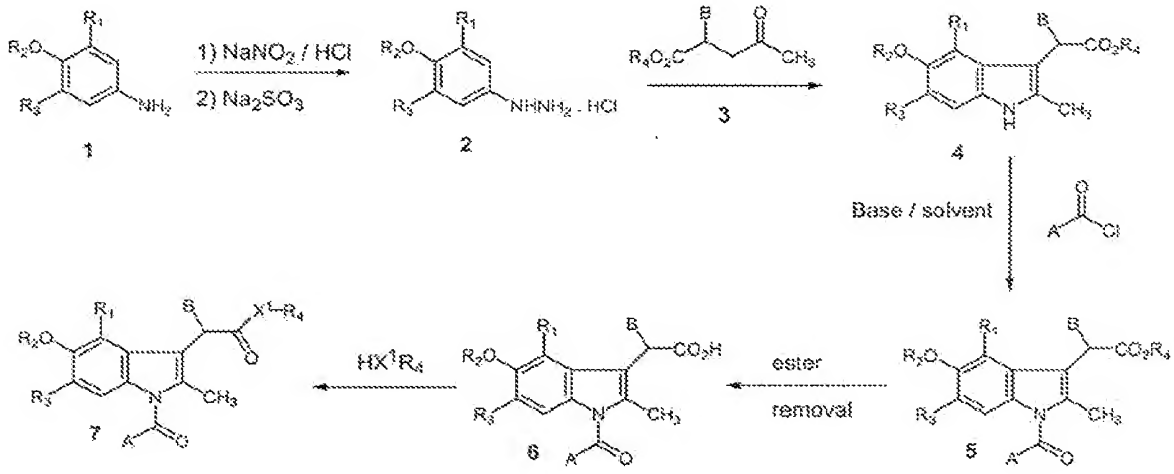


Demuynck, M.; DeClercq, P.; Vanderwalle, M. *J. Org. Chem.* **1979**, *44*. 4863-4866.
Grieco, P. A.; Nishizawa, M.; Oguri, T.; Burke, S. D.; Marinovic, N. *J. Am. Chem. Soc.* **1977**, *99*, 5773-5780.

Section 2

Preparation of {1-[(5-chlorothien-2-yl)carbonyl]-6-fluoro-5-hydroxy-2-methyl-1*H*-indol-3-yl}acetic acid

The preparation of this compound can be achieved as follows.



Step 1. Preparation of (3-fluoro-4-methoxyphenyl)hydrazine (**2**, R_1 = H, R_2 = CH_3, R_3 = F).

3-Fluoro-4-methoxyaniline (**1**, R_1 = H, R_2 = CH_3, R_3 = F) (95 g, 0.67 mol) was added to concentrated hydrochloric acid (250 mL), the suspension was stirred at ambient temperature for 18 hours, then it was cooled to 0 °C and a solution of sodium nitrite (53.7 g, 0.78 mol) in water (200 mL) was added dropwise at 0-5 °C. When the addition was complete, the resulting solution was stirred at 0 °C for 1 hour then it was added dropwise at 0-5 °C to a stirred solution of tin (II) chloride dihydrate (638.9 g, 2.83 mol) in concentrated hydrochloric acid (500 mL). The mixture was allowed to warm to ambient temperature then it was stored at 4 °C for 18 hours. The resulting precipitate was collected by filtration, washed with water (400 mL), and ether (1000 mL) and dried *in*

vacuo. The solid hydrochloride salt was basified by addition to 10% aqueous sodium hydroxide solution (800 mL), the free base was extracted into ether (2 X 400 mL), and the combined extracts were dried ($MgSO_4$) and the solvent removed *in vacuo* to give (3-fluoro-4-methoxyphenyl)hydrazine (**2**, R_1 = H, R_2 = CH_3, R_3 = F) (51.9 g, 50%) as a yellow solid, mp 46-50 °C; ^{1}HNMR ($CDCl_3$/250 MHz): 1.5 (s, 1H, N*H*-NH_2), 3.85 (s, 3H, OC*H*$_3$), 5.0 (s, 2H, NH-N*H*$_2$), 6.44 (m, 1H, phenyl 6-*H*), 6.60 (dd, 1H, phenyl 5-*H*), 6.79 (t, 1H, phenyl 2-*H*).

Step 2A. Preparation of (6-fluoro-5-methoxy-2-methyl-*1H*-indol-3-yl)acetic acid (**4**, R_1 = H, R_2 = CH_3, R_3 = F, R_4 = B = H) and (4-fluoro-5-methoxy-2-methyl-*1H*-indol-3-yl)acetic acid (**4**, R_1 = F, R_2 = CH_3, R_3 = H, R_4 = B = H).

Levulinic acid (**3**, B = R_4 = H) (38 mL, 354 mmol) and 3-fluoro-6-methoxy-phenylhydrazine hydrochloride (**2**, R_1 = H, R_2 = CH_3, R_3 = F) (67.5 g, 350 mmol) were combined and 150 mL of glacial acetic acid added and the slurry was stirred at 80 °C for 4 hours. The reaction was cooled to room temperature and added to ice water (500 mL). The resulting aqueous solution was extracted with dichloromethane (3 X 500 mL) and the organics dried ($MgSO_4$) and concentrated to afford a thick semi-solid. Water (450-500 mL) was added and the slurry was stirred vigorously overnight while manually breaking up large solid pieces with a spatula. The fine tan solid that resulted was isolated by filtration and dried to afford a mixture of indoles 56.3 grams, 67% yield, ~ 93% pure by HPLC (7/1 ratio of (6-fluoro-5-methoxy-2-methyl-*1H*-indol-3-yl)acetic acid (**4**, R_1 = H, R_2 = CH_3, R_3 = F, R_4 = B = H) and (4-fluoro-5-methoxy-2-methyl-*1H*-indol-3-yl)acetic acid (**4**, R_1 = F, R_2 = CH_3, R_3 = H, R_4 =B = H) of by NMR). Major isomer ^{1}H-NMR ($CDCl_3$/300 MHz) 2.27 (s, 3H), 3.82 (s, 2H), 3.84 (s, 3H), 6.92-6.97 (m, 2H, ArH).

Step 2B. Preparation of 2-trimethylsilylethyl (6-fluoro-5-methoxy-2-methyl-*1H*-indol-3-yl)acetate (**4**, R_1 = B = H, R_2 = CH_3, R_3 = F, R_4 = $CH_2CH_2Si(CH_3)_3$).

The indoles from Step 2A (56.3 g, 238 mmol) were combined with 2-trimethylsilylethanol (41 mL, 1.25 eq.) and 4-(dimethylamino)pyridine (DMAP) (4 g) in

dichloromethane (600 mL) and cooled to 0 °C. 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI) (50.2 g, 1.1 eq.) was added in portions and the reaction was stirred for 30 minutes at 0 °C and then allowed to warm to room temperature and stir overnight. The reaction mixture was diluted with dichloromethane (600 mL) and washed with water (2 X 200 mL), dried and concentrated to give a thick orange syrup which after triturating with hexanes induced solid formation, the solid was recrystallized from hexane-ethyl acetate to afford tan needles of 2-trimethylsilylethyl (6-fluoro-5-methoxy-2-methyl-*1H*-indol-3-yl)acetate (**4**, R_1 = H = B, R_2 = CH_3, R_3 = F, R_4 = $CH_2CH_2Si(CH_3)_3$), 52 g, 65% yield, >98% pure; ^{1}H-NMR ($CDCl_3$/300 MHz) 0.16 (s, 9H), 0.98 (m, 2H), 2.37 (s, 3H), 3.61 (s, 2H), 3.93 (s, 3H), 4.12 (m, 2H), 7.00-7.05 (m, 2H, ArH). The other regioisomer, 2-trimethylsilylethyl (4-fluoro-5-methoxy-2-methyl-*1H*-indol-3-yl)acetate (**4**, R_1 = F, R_3 = B = H, R_2 = CH_3, R_4 = $CH_2CH_2Si(CH_3)_3$), may be isolated by concentration of the filtrate and purification by chromatography on silica gel.

Step 3. Preparation of 2-trimethylsilylethyl-{1-[(5-chlorothien-2-yl)carbonyl]-6-fluoro-5-methoxy-2-methyl-*1H*-indol-3-yl}acetate (**5**, R_1 = H = B, R_2 = CH_3, R_3 = F, R_4 = $CH_2CH_2Si(CH_3)_3$, A = 5-chlorothiophene).

In a dry flask 2-trimethylsilylethyl (6-fluoro-5-methoxy-2-methyl-*1H*-indol-3-yl)acetate (**4**, R_1 = H = B, R_2 = CH_3, R_3 = F, R_4 = $CH_2CH_2Si(CH_3)_3$), (1.0 g, 2.96 mmol) was dissolved in tetrahydrofuran (THF) (10 mL) and hexamethylphosphoramide (HMPA) (1 mL) and cooled to –78 °C. Potassium bis(trimethylsilyl)amide 0.5M in toluene (6.52 mL) was added and the reaction was stirred for 30 minutes. 5-Chlorothiophene-2-carbonyl chloride (562 mg, 3.1 mmol) in 3 mL of THF was added and the reaction was stirred for 0.5 hours at –78 °C and 0.5 hours at 0 °C, and then treated with saturated ammonium chloride (20 mL) and the reaction extracted with ethyl acetate (3 x 30 mL), dried over $MgSO_4$ and concentrated to give a thick oil which was purified by chromatography to afford 2-trimethylsilylethyl-{1-[(5-chlorothien-2-yl)carbonyl]-6-fluoro-5-methoxy-2-methyl-*1H*-indol-3-yl}acetate (**5**, R_1 = H = B, R_2 = CH_3, R_3 = F, R_4 = $CH_2CH_2Si(CH_3)_3$, A = 5-chlorothiophene). (600 mg, 1.24 mmol, 42%, >99% pure) as light yellow oil; ^{1}H-NMR ($CDCl_3$/300 MHz) consistent with the assigned structure.

Step 4. Preparation of {1-[(5-chlorothien-2-yl)carbonyl]-6-fluoro-5-methoxy-2-methyl-*1H*-indol-3-yl}acetic acid (**6**, R_1 = H, R_2 = CH_3, R_3 = F, R_4 = H, A = 5-chlorothiophene).

A solution of the product from Step 3, 2-trimethylsilylethyl-{1-[(5-chlorothien-2-yl)carbonyl]-6-fluoro-5-methoxy-2-methyl-*1H*-indol-3-yl}acetate **5**, R_1 = H = B, R_2 = CH_3, R_3 = F, R_4 = $CH_2CH_2Si(CH_3)_3$, A = 5-chlorothiophene) (600 mg, 1.24 mmol) dissolved in 8 mL of THF was treated with a solution of tetrabutylammonium fluoride (1M, 3.1 mL, 3.1 mmol) in THF. The solution was stirred at room temperature until the ester had been cleaved (ca. 1 hour), and then the solution was diluted with saturated aqueous ammonium chloride and extracted with ethyl acetate. The combined extracts were washed with brine, dried over $MgSO_4$ and concentrated to give a solid that was purified by chromatography eluting with hexanes and ethyl acetate to provide 280 mg, 59% of pure {1-[(5-chlorothien-2-yl)carbonyl]-6-fluoro-5-methoxy-2-methyl-*1H*-indol-3-yl}acetic acid (**6**, R_1 = H, R_2 = CH_3, R_3 = F, R_4 = H, A = 5-chlorothiophene), mp 169 °C ^{1}H NMR ($CDCl_3$/300 MHz) 7.35 (d, 1H, *J* = 4.0 Hz), 7.09 (d, 1H, *J* = 11.7 Hz), 7.00 (d, 1H, *J* = 7.2 Hz), 6.98 (d, 1H, *J* = 4.0 Hz), 3.93 (s, 3H), 3.70 (s, 2H), 2.42 (s, 3H).

Step 5. Preparation of {1-[(5-chlorothien-2-yl)carbonyl]-6-fluoro-5-hydroxy-2-methyl-*1H*-indol-3-yl}acetic acid (**6**, R_1 = H = B, R_2 = H, R_3 = F, A = 5-chlorothiophene).

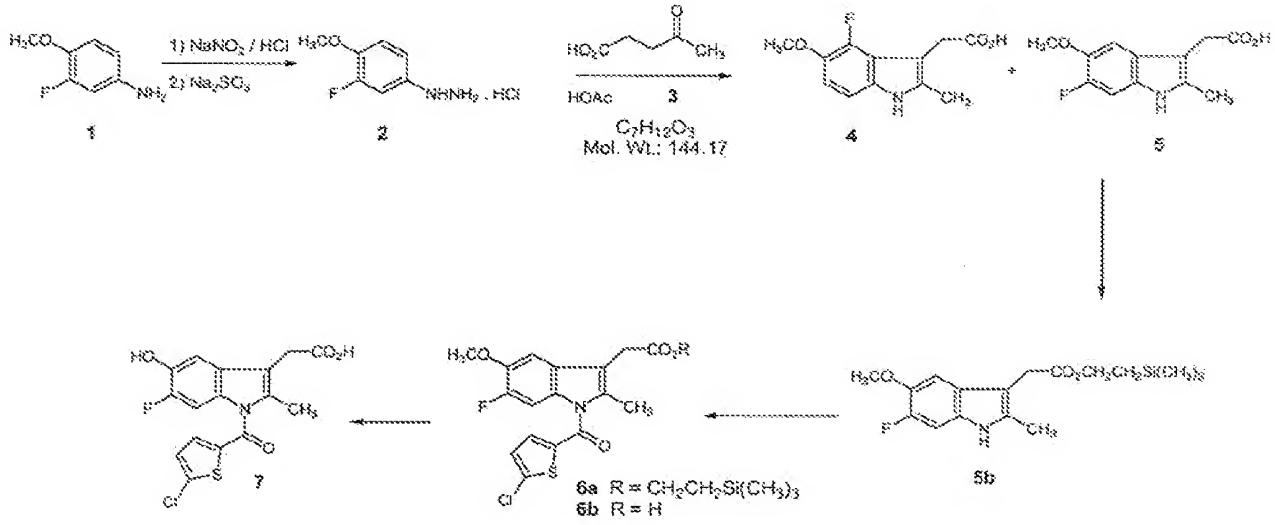
The product from Step 3, 2-trimethylsilylethyl-{1-[(5-chlorothien-2-yl)carbonyl]-6-fluoro-5-methoxy-2-methyl-*1H*-indol-3-yl}acetate **5**, R_1 = H = B, R_2 = CH_3, R_3 = F, R_4 = $CH_2CH_2Si(CH_3)_3$, A = 5-chlorothiophene) (400 mg, 0.83 mmol) was dissolved in 10 mL of dry dichloromethane and cooled to –78 °C. The solution was then treated with boron tribromide (1M, 4.9 mL, 4.9 mmol) in dichloromethane and the solution allowed to warm to room temperature and stirred at that temperature for an additional 2 hours. The solution was then poured into water and the phases separated and the aqueous phase extracted with dichloromethane. The combined extracts were washed with brine, dried

over $MgSO_4$ and concentrated to give a solid that was purified by chromatography eluting with methanol and dichloromethane to provide 150 mg, 49%, of pure {1-[(5-chlorothien-2-yl)carbonyl]-6-fluoro-5-hydroxy-2-methyl-*1H*-indol-3-yl}acetic acid (**6**, R_1 = H = B, R_2 = H, R_3 = F, A = 5-chlorothiophene) mp 174 °C, ^{1}H NMR ($CDCl_3$/300 MHz) 7.34 (d, 1H, *J* = 3.9 Hz), 7.13 (d, 1H, *J* = 11.1 Hz), 7.07 (d, 1H, *J* = 8.4 Hz), 6.98 (d, 1H, *J* = 3.9 Hz), 3.66 (s, 2H), 2.39 (s, 3H).

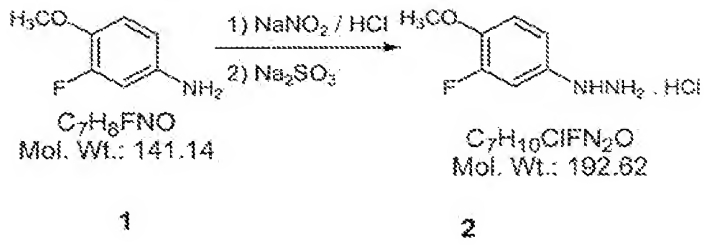
Section 3

General Synthesis Scheme 1

Certain useful compounds may be prepared by the general method outlined in below.



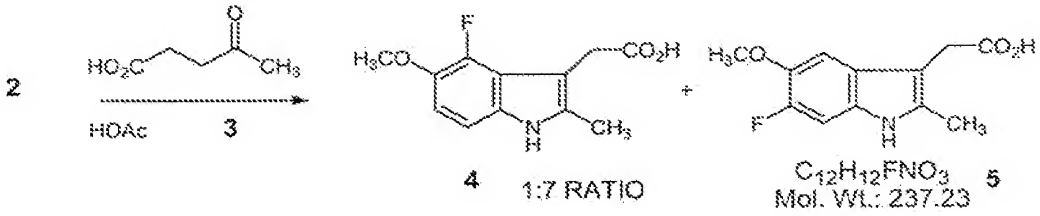
Step 1. Preparation of phenylhydrazines, representative example: (3-fluoro-4-methoxyphenyl)hydrazine (**2**).



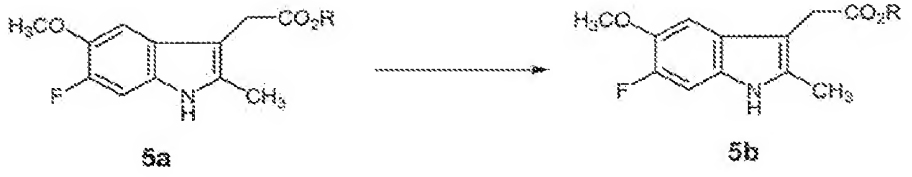
1 2

The preparation of phenylhydrazine derivatives (**2**) begins with treatment of commercially available anilines (**1**) with nitrous acid, generated from sodium nitrite and concentrated hydrochloric acid, to produce the corresponding diazonium salt. In the same reaction vessel the diazonium salt is treated with sodium sulfite and hydrochloric acid to produce the desired hydrazine hydrochloride (**2**) in 90% yield. Alternatively, the diazonium salt can be reduced with stannous chloride in hydrochloric acid.

Step 2. Preparation of indoles by the Fisher Indole synthesis, representative example: (6-fluoro-5-methoxy-2-methyl-*1H*-indol-3-yl)acetic acid (**5**).



Condensation of hydrazine hydrochloride (**2**) with levulinic acid (**3**) in acetic acid results in the formation of two regioisomeric indole derivatives **4** and **5** in a 1:7 ratio. The major regioisomer **5** can be isolated in pure form by crystallization of the reaction mixture. Alternatively, the indole mixture can be esterified with an alcohol such as 2-trimethylsilylethanol to afford the corresponding esters that can then be separated by a number of means, for example by chromatography.



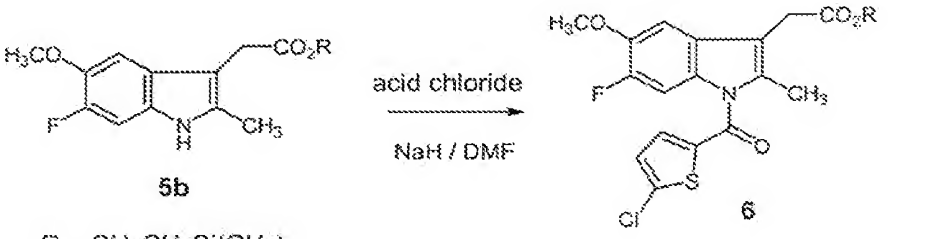
5a

R = H
$C_{12}H_{12}FNO_3$
Mol. Wt.: 237.23

5b

R = $CH_2CH_2Si(CH_3)_3$
$C_{17}H_{24}FNO_3Si$
Mol. Wt.: 337.46

Step 3. Acylation of indole **5b**: preparation of 2-trimethylsilylethyl-{1-[(5-chlorothien-2-yl)carbonyl]-6-fluoro-5-methoxy-2-methyl-*1H*-indol-3-yl}acetate (**6b**).



5b

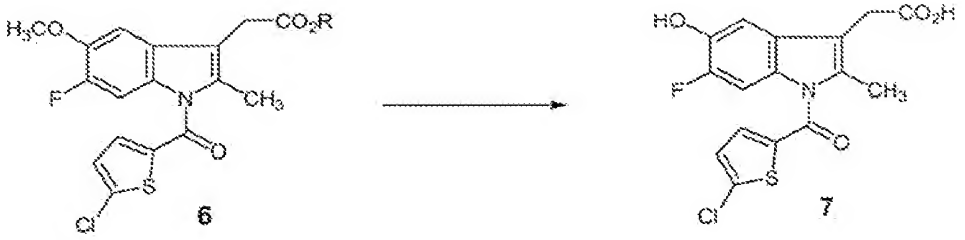
R = $CH_2CH_2Si(CH_3)_3$
$C_{17}H_{24}FNO_3Si$
Mol. Wt.: 337.46

6

a) R = H
b) R = $CH_2CH_2Si(CH_3)_3$

Treatment of the indole ester **5b** with sodium hydride in dimethylformamide (DMF) followed by treatment with an acid chloride such as 5-chlorothiophene-2-carbonyl chloride affords the acylated indole derivative **6b** in 82% yield. The ester can then be removed by treatment with an acid such as trifluoroacetic acid to produce the corresponding acid, in this instance **6a**.

Step 4. Preparation of 5-hydroxy indole derivatives; preparation of {1-[(5-chlorothien-2-yl)carbonyl]-6-fluoro-5-methoxy-2-methyl-*1H*-indol-3-yl}acetic acid (7).

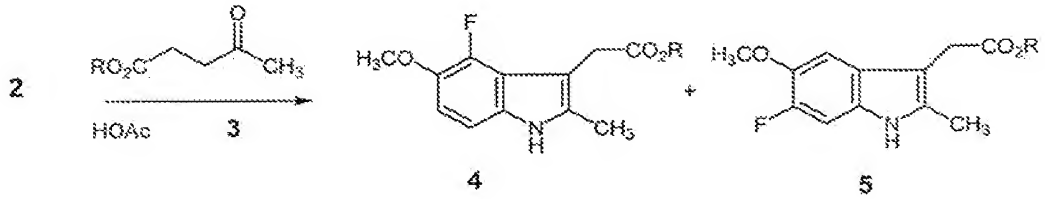


a) R = H
b) R = $CH_2CH_2Si(CH_3)_3$

Esters such as **6b** upon treatment with excess boron tribromide in dichloromethane can be converted to the corresponding acid phenols, such as **7** in good yield. Under these reaction conditions both the ester and the 5-methoxy moieties are dealkylated to the acid and phenol respectively. If desired the carboxylic acids can be converted to their salt derivatives by treatment with a base such as sodium hydroxide.

General Synthesis Scheme 2

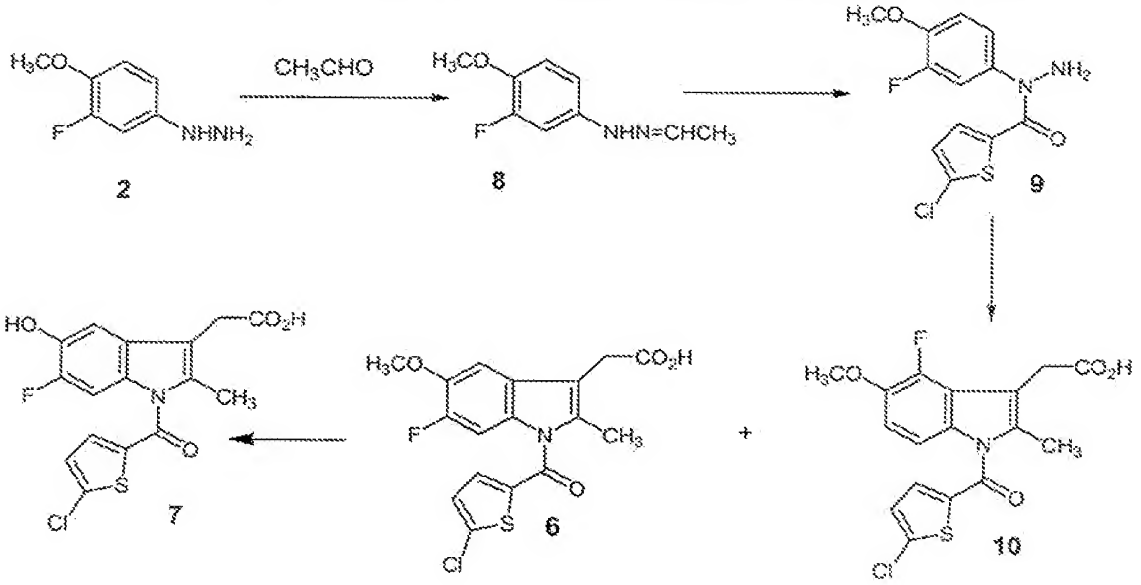
Certain compounds can be prepared according to general synthesis scheme 2 as follows.



In the first step the hydrazine (**2**) is condensed with and ester of levulinic acid in acetic acid to afford a mixture of regioisomeric indole esters **4** and **5** (for example if one uses ethyl levulinate (**3**, R = Et the products (**4** and **5**) will be the ethyl esters, R = Et). The esters can be separated and then acylated by the procedure outline in Scheme 1 to afford the corresponding acyl derivatives such as **6**, R = Et in the present example. Hydrolysis of the ester affords the corresponding acid, **6a**. If desired, the ester and the 5-methoxy groups can be removed in a single operation upon treatment with boron tribromide in dichloromethane to give phenols such as **7**.

General Synthesis Scheme 3

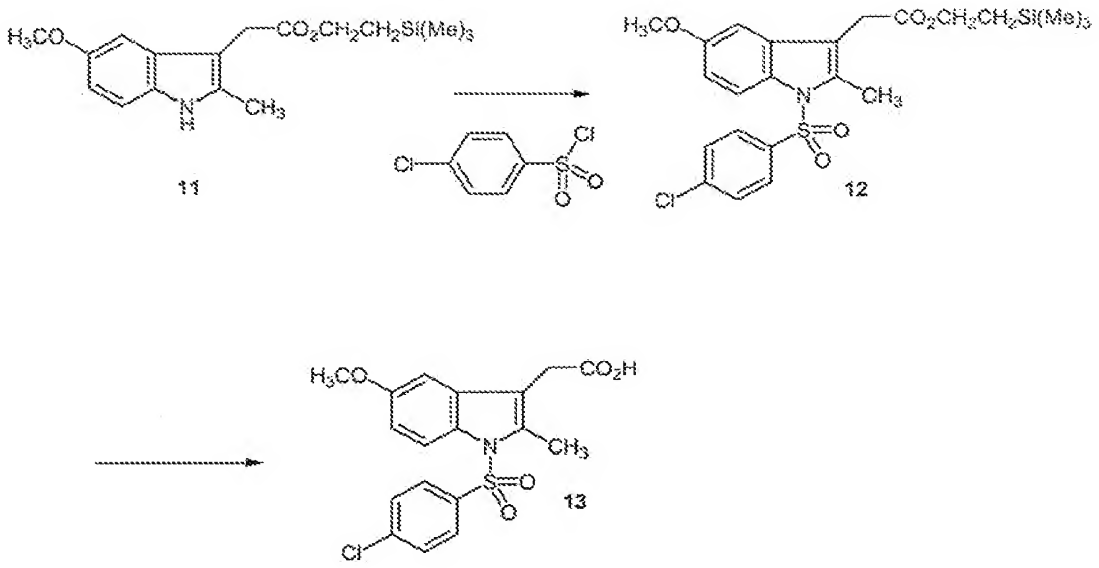
Certain compounds can be prepared according to general synthesis scheme below



The route commences with the condensation of phenylhydrazine derivatives such as **2** with acetaldehyde to afford the corresponding hydrazone **8**. Acylation of **8** with an acid chloride, in the present example 5-chlorothiophene-2-carbonyl chloride, followed by treatment with gaseous hydrochloric acid in an alcohol such as methanol provides the desired acylated hydrazine **9** after neutralization of the excess acid. Condensation of **9** with levulinic acid provides a mixture of regioisomers that can then be separated to afford acylated indoles, in the present example, **6** and **10**. If desired, the 5-methoxy group can then be converted to the corresponding 5-hydroxy substituent by treatment with boron tribromide in dichloromethane for example to prepare **7**.

General Synthesis Scheme 4

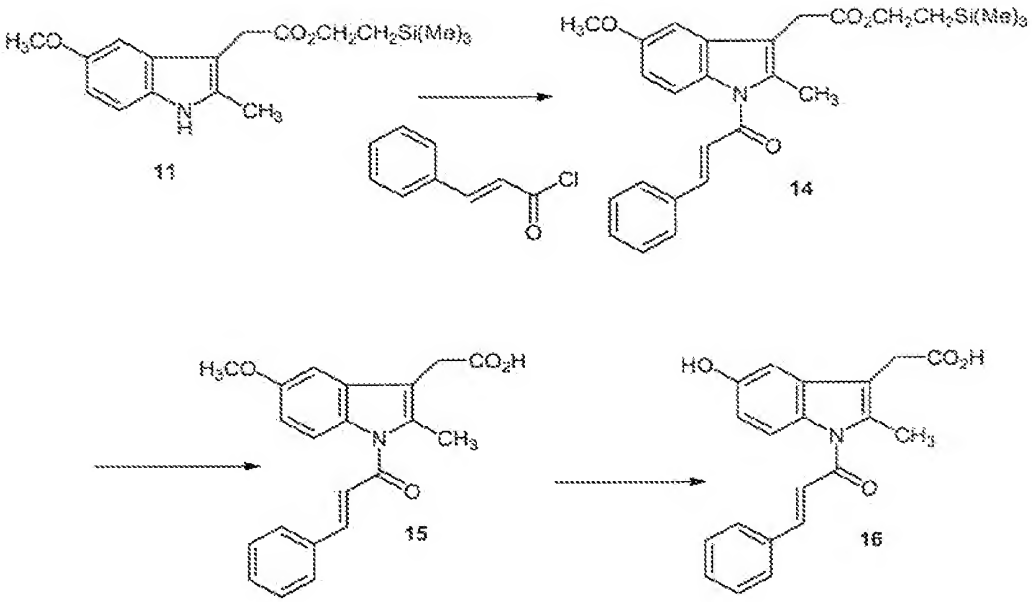
Certain compounds can be prepared according to general synthesis scheme 4 as follows.



Treatment of the indole ester **11**, prepared from the corresponding indole acid by coupling with 2-trimethylsilylethanol in the presence of a dehydrating agent such as dicyclohexylcarbodiimide, with a strong base such as potassium bis(trimethylsilyl)amide in tetrahydrofuran generates the indole anion that can be condensed with a sulfonyl chloride to afford the *N*-sulfonyl derivatives such as **12**. In the present example 4-chlorobenzenesulfonyl chloride was used the sulfonyl chloride. In the second step the *N*-sulfonyl indole **12** is converted into the corresponding indole acid **13** upon treatment with tetrabutylammonium fluoride in tetrahydrofuran. If desired, the 5-methoxy substituent can be converted to the corresponding 5-hydroxy group upon treatment of **13** with boron tribromide in dichloromethane.

General Synthesis Scheme 5

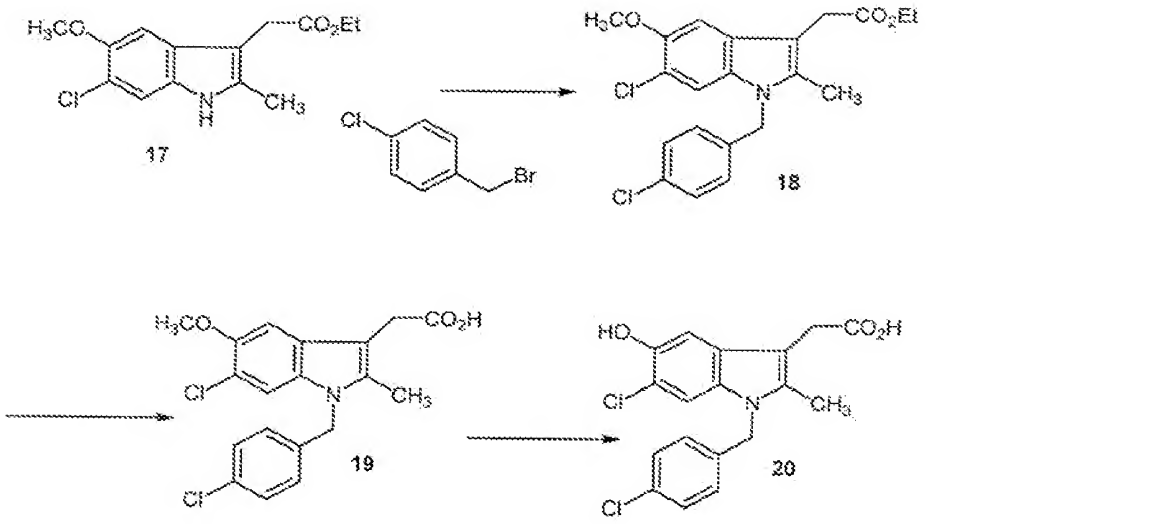
Certain compounds can be prepared according to general synthesis scheme 5 as follows



Treatment of the indole ester **11**, prepared from the corresponding indole acid by coupling with 2-trimethylsilylethanol in the presence of a dehydrating agent such as dicyclohexylcarbodiimide, with a strong base such as potassium bis(trimethylsilyl)amide in tetrahydrofuran generates the indole anion that can be condensed with a cinnamoyl chloride to afford the *N*-acyl derivative **14**. In the second step the *N*-acyl indole **14** is converted into the corresponding indole acid **15** upon treatment with tetrabutylammonium fluoride in tetrahydrofuran. If desired, the 5-methoxy substituent can be converted to the corresponding 5-hydroxy group, **16**, upon treatment of **15** with boron tribromide in dichloromethane.

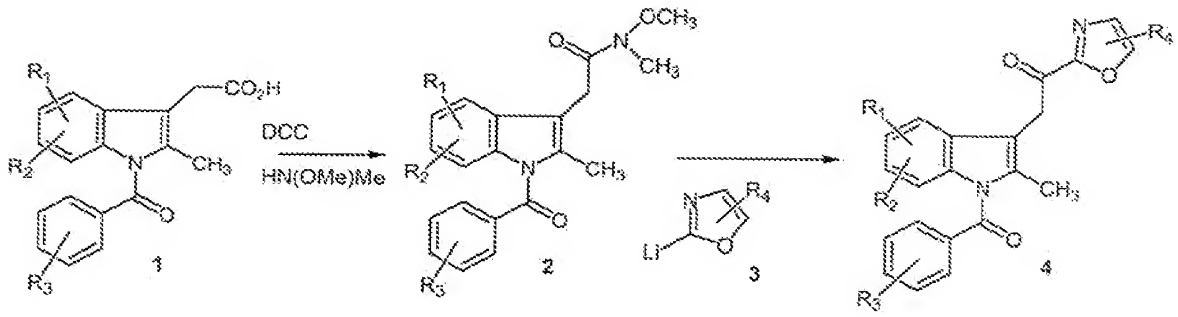
General Synthesis Scheme 6

Certain compounds can be prepared according to general synthesis scheme 6 as follows



Treatment of the indole ester **17** with a strong base such as potassium bis(trimethylsilyl)amide in tetrahydrofuran generates the indole anion that can be alkylated with 4-chlorobenzyl bromide to afford the *N*-benzyl derivative **18**. In the second step the *N*-benzyl indole **18** is converted into the corresponding indole acid **19** upon treatment with sodium hydroxide in aqueous tetrahydrofuran. If desired, the 5-methoxy substituent can be converted to the corresponding 5-hydroxy group, **20**, upon treatment of **19** with boron tribromide in dichloromethane.

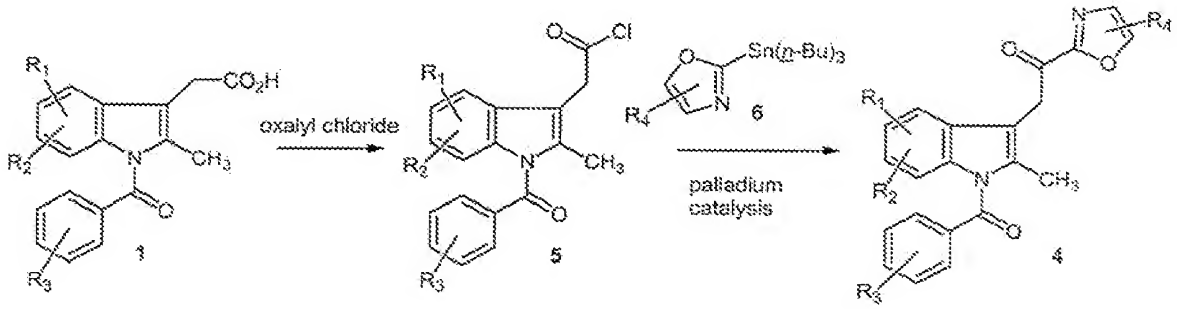
General Synthesis Scheme 7



Illustrated in General Synthesiis Scheme 7 is the general method for the preparation of FAAH inhibitor compounds of the general formula (**4**). The synthesis commences with the condensation of an indole carboxylic acid derivative **1** with *N,O*-

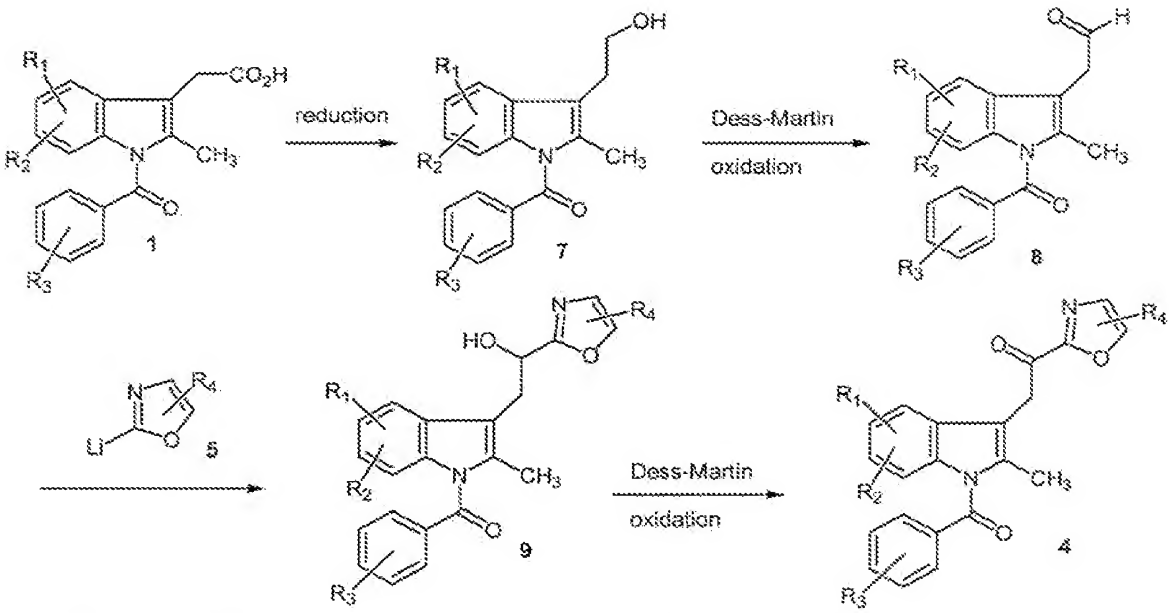
dimethylhydroxylamine (generated from *N,O*-dimethylhydroxylamine hydrochloride with triethylamine) in the presence of a dehydrating agent such as dicyclohexylcarbodiimide (DCC) to provide the so-called Weinreb amide derivative **2**. Treatment of amide **2** with the lithium derivative **3**, generated by treatment of the corresponding oxazole with *n*-butyllithium, provides the ketooxazole derivatives of formula **4**.

General Synthesis Scheme 8



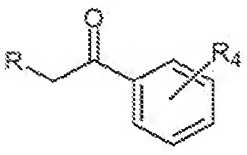
An alternate procedure for the preparation of compounds of the general formula **4** is illustrated in General Synthesis Scheme 8. The indole carboxylic acid derivative **1** is converted to the corresponding acid chloride **5** by treatment with oxalyl chloride or with thionyl chloride ($SOCl_2$). The acid chloride is then treated with an organostannane such as **6** in the presence of palladium catalysts to affect Stille coupling. If necessary this later reaction can be conducted under an atmosphere of carbon monoxide to suppress decarbonylation of the acid chloride during the Stille coupling. In a variation on the above scheme the organolithium derivative **3** can be converted to the corresponding organozinc derivative ($ZnCl_2$) and then coupled to acid chloride **5** to produce the compounds of formula **4**.

General Synthesis Scheme 9

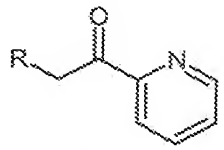


In certain instances it may be advantageous to use the route outlined in General Synthesis Scheme 9. This method commences with the reduction of the carboxylic acid moiety of **1** to afford the corresponding alcohol **7** and subsequent conversion to the corresponding aldehyde **8** under suitable oxidation conditions such as with the Dess-Martin periodinane reagent. The aldehyde **8** can then be treated with the organolithium reagent **5** to prepare alcohol **9**, which in turn is converted into the desired ketones **4** by oxidation with the Dess-Martin periodinane reagent.

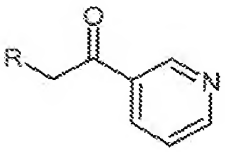
Using the same general methods outlined in General Synthesis Schemes 7, 8 and 9 it is possible to prepare a wide range of ketone derivatives by changing the nature of the organolithium, organozinc or organotin derivative. The following generalized examples show how each of the desired ketones can be prepared from the appropriate starting materials.



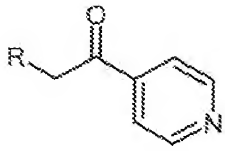
Prepared from the appropriate phenyllithium derivative and the Weinreb amide such as **2**.



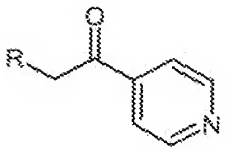
Prepared from 2-bromopyridine by treatment with *n*-butyllithium and condensation with a Weinreb amide such as **2**. Alternatively, 2-bromopyridine can be converted into 2-(tri-*n*-butylstannanyl)pyridine and then condensed with an acid chloride such as **5** under palladium catalysis.



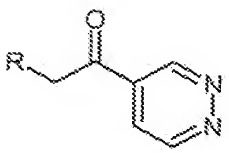
Prepared from 3-bromopyridine by treatment with *n*-butyllithium and condensation with a Weinreb amide such as **2**. Alternatively, 3-bromopyridine can be converted into 3-(tri-*n*-butylstannanyl)pyridine and then condensed with an acid chloride such as **5** under palladium catalysis.



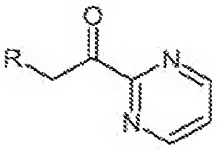
Prepared from 4-bromopyridine by treatment with *n*-butyllithium and condensation with a Weinreb amide such as **2**. Alternatively, 4-bromopyridine can be converted into 4-(tri-*n*-butylstannanyl)pyridine and then condensed with an acid chloride such as **5** under palladium catalysis.



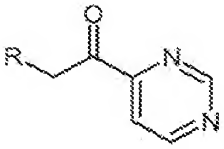
Prepared from pyridazine by treatment with excess lithium tetramethylpiperidide and condensation with a Weinreb amide such as **2**. Alternatively, treatment of 3(2*H*)-pyridazinone with trifluoromethanesulfonic anhydride followed by coupling with hexabutylditin promoted by palladium catalysis affords 3-(tri-*n*-butylstannyl)pyridazine which can then be condensed with an acid chloride such as **5** under palladium catalysis.



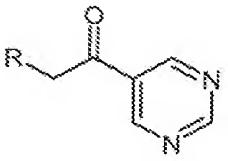
4-(tri-*n*-Butylstannyl)pyridazine can be condensed with an acid chloride such as **5** under palladium catalysis to afford these desired ketones.



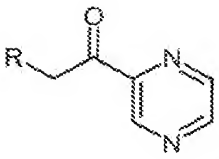
Reaction of tri-*n*-butylstannyl lithium with 2-chloropyrimidine affords 2-(tributylstannyl)pyrimidine which can be condensed the an acid chloride such as **5** under palladium catalysis to afford these desired ketones.



4(3*H*)-Pyrimidone upon treatment with trifluoromethanesulfonic anhydride in pyridine provides the corresponding triflate that upon reaction with hexabutylditin in the presence of *bis*-(triphenylphosphine)palladium (II) chloride produces 4-(tributylstannyl)pyrimidine. Treatment of 4-(tributylstannyl)pyrimidine with an acid chloride such as **5** under palladium catalysis affords these desired ketones.

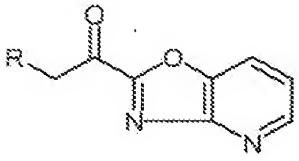


Treatment of 5-bromopyrimidine with hexabutylditin in the presence of a palladium catalyst affords 5-(tributylstannyl)pyrimidine which can be treated with an acid chloride such as **5** to afford these desired ketones.

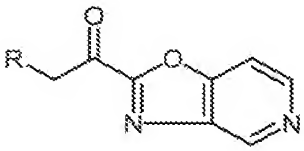


2-(Tributylstannyl)pyrazine prepared by treatment of chloropyrazine with *n*-butyllithium followed by condensation with tri-*n*-butyltin chloride is treated with

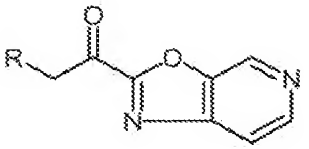
an acid chloride such as **5** to afford the desired ketones. Alternatively, the lithium derivative derived from treatment of chloropyrazine with *n*-butyllithium can be condensed directly with the Weinreb amides such as **2** to prepare these desired ketones.



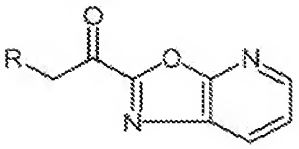
Treatment of aldehydes such as **8** with [1,3]oxazolo[5,4-*b*]pyridin-2-yllithium followed by oxidation of the incipient alcohol with the Dess-Martin periodinane reagent can be used to prepare these desired ketones



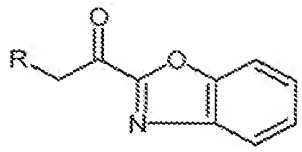
Treatment of aldehydes such as **8** with [1,3]oxazolo[5,4-*c*]pyridin-2-yllithium followed by oxidation of the incipient alcohol with the Dess-Martin periodinane reagent can be used to prepare these desired ketones.



Treatment of aldehydes such as **8** with [1,3]oxazolo[4,5-*c*]pyridin-2-yllithium followed by oxidation of the incipient alcohol with the Dess-Martin periodinane reagent can be used to prepare these desired ketones.

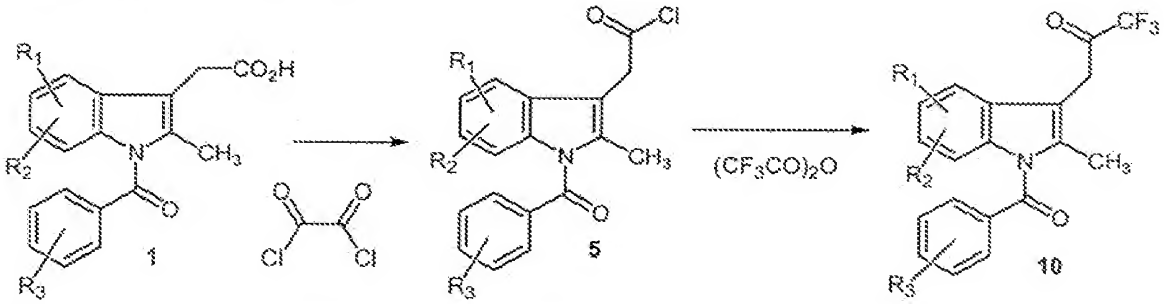


Treatment of aldehydes such as **8** with [1,3]oxazolo[4,5-*b*]pyridin-2-yllithium followed by oxidation of the incipient alcohol with the Dess-Martin periodinane reagent can be used to prepare these desired ketones.



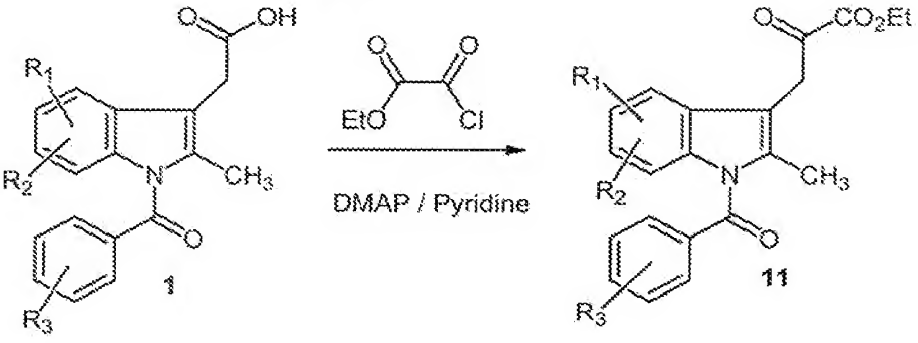
Treatment of aldehydes such as **8** with 1,3-benzoxazol-2-yllithium followed by oxidation of the incipient alcohol with the Dess-Martin periodinane reagent can be used to prepare these desired ketones.

General Synthesis Scheme 10



Illustrated in General Synthesis Scheme 10 is the method for the preparation of trifluoromethy ketones such as **10**. The carboxylic acids **1** are converted to the corresponding acid chloride **5** with oxalyl chloride and then converted into the trifluoromethyl ketones by treatment with trifluoroacetic anhydride in the presence pyridine according to the method described by Boivin, J.; El Kaim, L.; Zard, S. Z., *Tetrahedron Lett.* **1992**, *33*, 1285-1288.

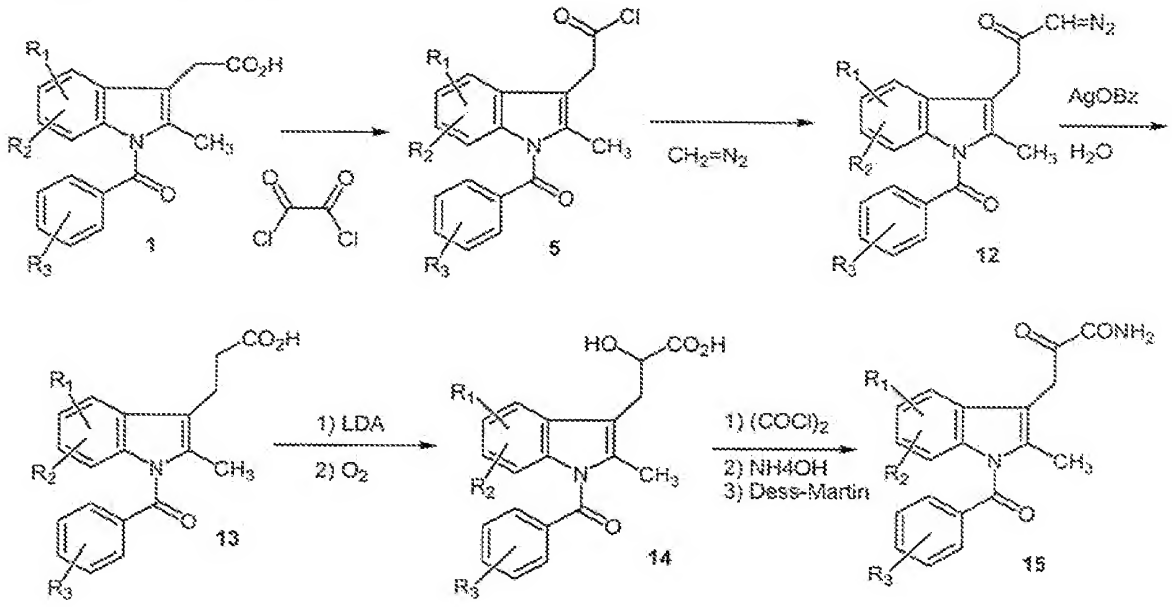
General Synthesis Scheme 11



Illustrated in General Synthesis Scheme 11 is the method that was used to prepare alpha-ketoester derivatives of the general formula **11**. The procedure involves condensation of

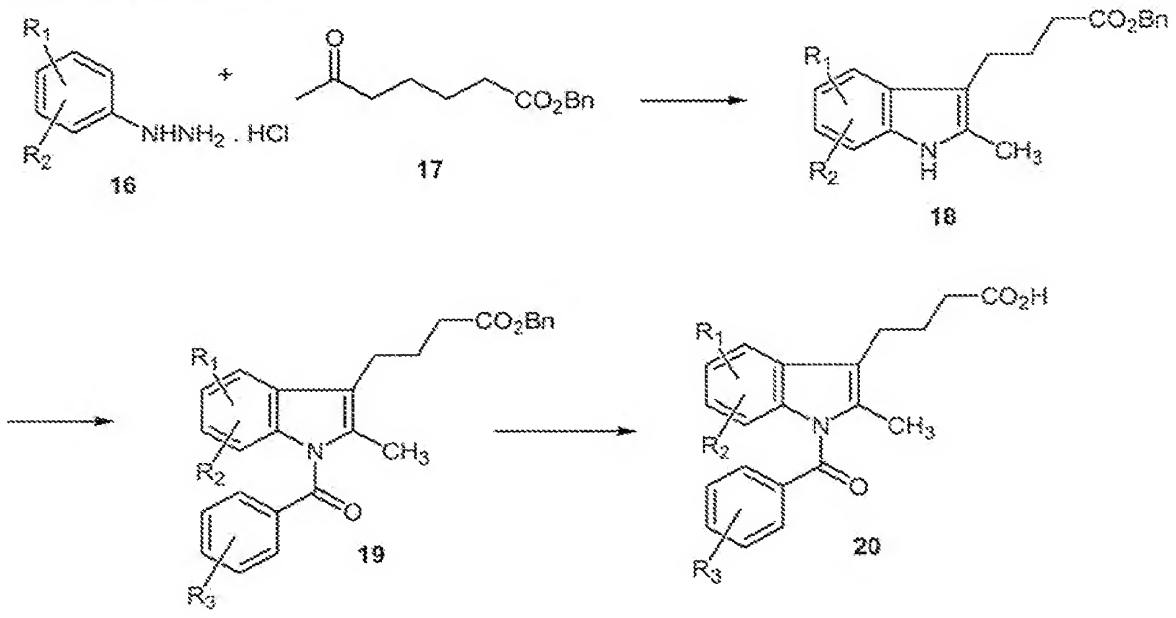
the carboxylic acids **1** with ethyl chlorooxoacetate in the presence of pyridine and 4-(dimethylamino)pyridine (DMAP) to produce these desired keto esters **11** according to the method of Li, Z.; Patil, G. S.; Golubski, Z. E.; Hori, H.; Tehrani, K.; Foreman, J. E.; Eveleth, D. D.; Bartus, R. T.; Powers, J. C., *J. Med. Chem.* **1993**, *36*, 3472-3480.

General Synthesis Scheme 12



Illustrated in General Synthesis Scheme 12 is the method that was used to prepare inhibitors of the general formula **13**-**15**. The sequence commences with the conversion of carboxylic acids **1** to the corresponding acid chlorides **5** with oxalyl chloride. Treatment of **5** with diazomethane affords the corresponding diazoketones **12** in excellent yield that can then be converted into the chain extended carboxylic acids **13** upon treatment with silver benzoate in aqueous tetrahydrofuran. Oxygenation of the enolate of **13** generated with lithium diisopropylamide provides the alpha-hydroxyacids **14**. If desired, the alpha-keto amides **15** can be generated from **14** by a sequence involving conversion to the acid chloride, treatment with aqueous ammonia, and finally oxidation of the alcohol to the corresponding ketones with the Dess-Martin periodinane reagent.

General Synthesis Scheme 13



General Synthesis Scheme 13 illustrates the method that can be used for the preparation of compounds of the general formula **20**. The process commences with the condensation of an aryl hydrazine derivative **16** with benzyl 6-oxoheptanoate (17) under the standard Fisher indole synthesis conditions to afford indole derivatives **18**. Acylation of the indole nitrogen with an acid chloride to provide the new indole derivatives **19** is normally accomplished by generation of the indole anion with a strong base such as sodium hydride. The benzyl ester of **19** is then removed by hydrogenolysis over palladium on carbon to afford the desired indole butyric acid derivatives **20**.

Section 4

Synthesis of Heterocylic Fused Ring Systems

Various methods are known for the synthesis of fused heterocyclic ring systems. Several are referenced below. Many others are known and useful.

[1,3]thiazolo[4,5-b]pyridine-2-yl can be prepared according to: WO2004058728.

[1,3]thiazolo[4,5-*c*]pyridine-2-yl can be prepared according to: International Journal of Sulfur Chemistry, Part B: Quarterly Reports on Sulfur Chemistry (1972), 7(2), 121-153.

[1,3]thiazolo[5,4-*c*]pyridine-2-yl can be prepared according to Journal of Heterocyclic Chemistry (1990), 27(3), 563-566.

[1,3]thiazolo[5,4-*b*]pyridine-2-yl can be prepared according to International Journal of Sulfur Chemistry, Part B: Quarterly Reports on Sulfur Chemistry (1972), 7(2), 121-153.

[1,3]thiazolo[4,5-*b*]pyrazine-2-yl can be prepared according to Science of Synthesis (2002), 11 835-912.

[1,3]thiazolo[4,5-*d*]pyrimidine-2-yl can be prepared according to Indian Journal of Chemistry (1971), 9(7), 651-654

[1,3]thiazolo[4,5-*d*]pyridazine-2-yl can be prepared according to Bulletin de la Societe Chimique de France (1971), (4), 1491-1496.

[1,3]oxazolo[4,5-*d*]pyrimidine-2-yl can be prepared according to Tetrahedron Letters (1990), 31(8), 1155-1157.

[1,3]oxazolo[5,4-*d*]pyrimidine-2-yl can be prepared according toAustralian Journal of Chemistry (1970), 23(6), 1229-1248.

[1,3]oxazolo[4,5-*b*]pyridine-2-yl can be prepared according to Heterocycles (1995), 41(3), 477-485.

[1,3]oxazolo[4,5-*c*]pyridine-2-yl can be prepared according to EP 1203766

[1,3]oxazolo[5,4-*c*]pyridine-2-yl can be prepared according to WO 2004064778

[1,3]oxazolo[5,4-*b*]pyridine-2-yl can be prepared according to WO 2003048137

Furo[2,3-*b*]pyridine-2-yl can be prepared according to Synthesis (1981), (6), 464-465.

Furo[2,3-*c*]pyridine-2-yl can be prepared according to Journal of Heterocyclic Chemistry (1982), 19(5), 1207-1209.

Furo[3,2-*c*]pyridine-2-yl can be prepared according to Journal of Heterocyclic Chemistry (1971), 8(1), 57-60.

Furo[3,2-*b*]pyridine-2-yl can be prepared according to Journal of Heterocyclic Chemistry (1986), 23(3), 665-668.

Thieno[3,2-*d*]pyrimidine-6-yl can be prepared according to Tetrahedron (1971), 27(2), 487-499.

Thieno[2,3-*d*]pyrimidine-6-yl can be prepared according to Journal of Heterocyclic Chemistry (1975), 12(5), 921-924.

Thieno[2,3-*c*]pyridazine-6-yl can be prepared according to Phosphorus, Sulfur and Silicon and the Related Elements (2004), 179(2), 321-344.

Thieno[2,3-*d*]pyridazine-2-yl can be prepared according to Bulletin de la Societe Chimique de France (1967), (7), 2495-2507.

Thieno[3,2-*c*]pyridazine-6-yl can be prepared according to Journal of the Chemical Society [Section] C: Organic (1971), (7), 1285-1291.

Thieno[2,3-*b*]pyrazine-6-yl can be prepared according to Journal of Heterocyclic Chemistry (1976), 13(2), 273-275.

Thieno[3,2-*b*]pyridine-2-yl can be prepared according to Journal of Heterocyclic Chemistry (1984), 21(3), 785-789.

Thieno[3,2-*c*]pyridine-2-yl can be prepared according to Journal of Heterocyclic Chemistry (1993), 30(1), 289-290.

Thieno[2,3-*c*]pyridine-2-yl can be prepared according to Synthesis (2004), (12), 1935-1937.

Thieno[2,3-*b*]pyridine-2-yl can be prepared according to Journal of Organic Chemistry (1987), 52(19), 4280-4287.

3*H*-imidazo[4,5-*b*]pyridine-2-yl can be prepared according to Tetrahedron Letters (1993), 34(12), 1897-1900.

3*H*-imidazo[4,5-*c*]pyridine-2-yl can be prepared according to Khimiya Geterotsiklicheskikh Soedinenii (1994), (10), 1411-1419.

7*H*-purine-8-yl can be prepared according to Chemische Berichte (1967), 100(7), 2280-2291.

1*H*-pyrrolo[3,2-*b*]pyridine-2-yl can be prepared according to Journal of Heterocyclic Chemistry (1992), 29(2), 359-367.

1*H*-pyrrolo[3,2-*c*]pyridine-2-yl can be prepared according to Heterocycles (1992), 34(12), 2379-2384.

1*H*-pyrrolo[2,3-*c*]pyridine-2-yl can be prepared according to Synthesis (1996), (7), 877-882.

1*H*-pyrrolo[2,3-*b*]pyridine-2-yl can be prepared according to Journal of the Chemical Society [Section] C: Organic (1969), (11), 1505-1514.

1*H*-pyrrolo[2,3-*d*]pyridazine-2-yl can be prepared according to Comptes Rendus des Seances de l'Academie des Sciences, Serie C: Sciences Chimiques (1967), 265(22), 1271-1273.

5*H*-pyrrolo[3,2-*c*]pyridazine-6-yl can be prepared according to Diss. Abstr. Int. B 1974, 35(3), 1199.

7*H*-pyrrolo[2,3-*c*]pyridazine-6-yl can be prepared according to Diss. Abstr. Int. B 1974, 35(3), 1199.

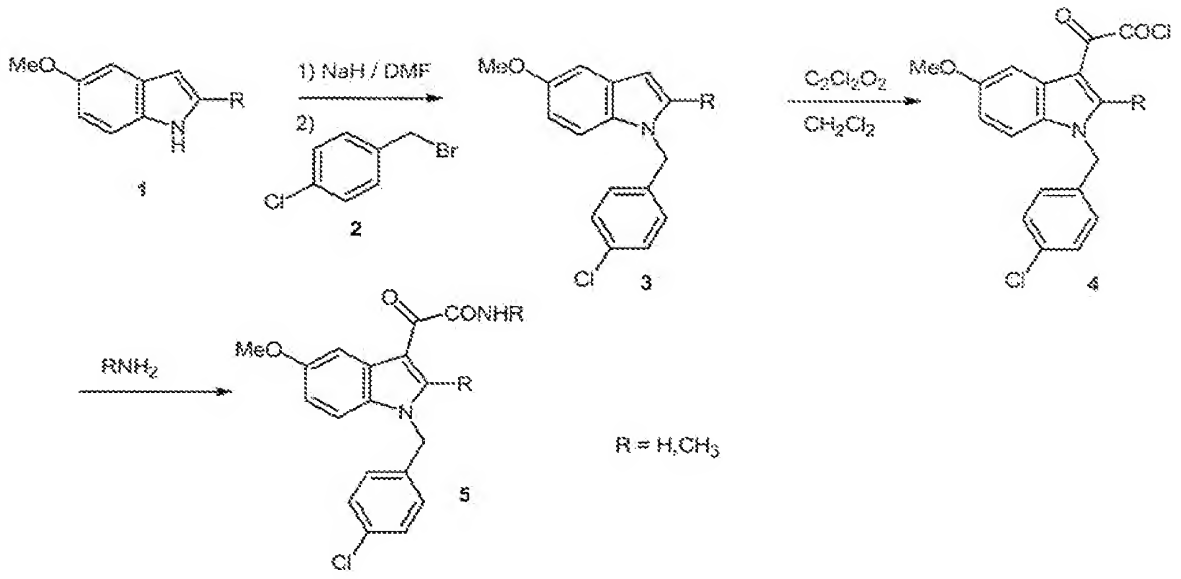
5*H*-pyrrolo[2,3-*b*]pyrazine-6-yl can be prepared according to Tetrahedron Letters (2004), 45(43), 8087-8090.

5*H*-pyrrolo[3,2-*d*]pyrimidine-6-yl can be prepared according to Synthesis (1974), (12), 837-859.

7*H*-pyrrolo[2,3-*d*]pyrimidine-6-yl can be prepared according to WO 2003048120,

Section 5

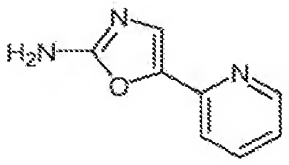
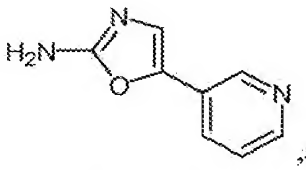
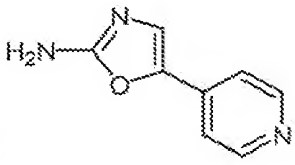
Preparation of alpha-keto amide derivatives.



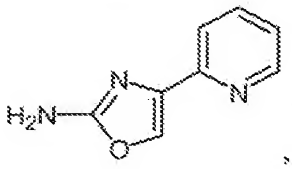
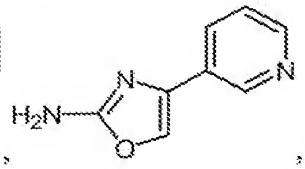
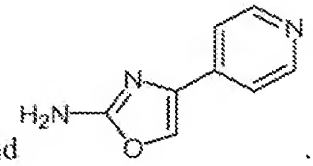
Treatment of indole derivative 1 with sodium hydride in dry dimethylformamide (DMF) and reaction with benzyl bromide 2 provides the alkylated derivative 3. Treatment of 3 with oxalyl chloride in dichloromethane affords acid chloride 4 that can be reacted with amines to provide amide derivatives with the general structure 5. Most of the amines are commercially available or can be prepared by methods described in the literature. These amines include those represented by CA Registry Numbers: 504-29-0 (Alfa Aesar, Ward Hill, MA, catalog no. A12374); 108-91-8 (Sigma-Aldrich, St. Louis, MO, catalog no. 240648), 765-30-0 (Sigma-Aldrich, St. Louis, MO, catalog no. 125504); 2516-34-9 (Alfa Aesar, Ward Hill, MA, catalog no. A13423); 1003-03-8 (Alfa Aesar, Ward Hill, MA, catalog no. L01966); 96-50-4 (Alfa Aesar, Ward Hill, MA, catalog no. A12026); 7720-39-0; 4570-45-0 (GLSynthesis Inc., Worcester, MA); 462-08-8 (Sigma-Aldrich, St. Louis, MO); 504-24-5; 591-54-8 (Sigma-Aldrich, St. Louis, MO, catalog no. 261823); 5049-61-6 (Alfa Aesar, Ward Hill, MA, catalog no. A13052); 109-12-6 (Alfa Aesar, Ward Hill, MA, catalog no. B24594); 5469-70-5 (see Turck, et al. Tetrahedron 1993, 49, 599-606); 20744-39-2 (SYNCHEM OHG, Kassel, Germany, catalog no. ct267); 591-55-9; 14678-05-8 (see Iwai and Nakamura, Chemical & Pharmaceutical Bulletin 1966, 14, 1277-1286); 1750-42-1 (Sigma-Aldrich, St. Louis, MO, catalog no. 424218); 14678-02-5 (Sigma-Aldrich, St. Louis, MO, catalog no. 304271); 1072-67-9 (Sigma-Aldrich, St. Louis, MO, catalog no. 232270); 1820-80-0 (Sigma-Aldrich, St. Louis, MO, catalog no.

160644); 31230-17-8 (Alfa Aesar, Ward Hill, MA, catalog no. A11642); 4592-62-5 (see Brown and Sainsbury, Science of Synthesis 2002, 11 507-572); 82357-92-4 (see Goerdeler and Pohland, Angew. Chem. 1960, 72, 77); 128146-85-0; 24340-76-9 (see Samaritoni et al, Journal of Agricultural and Food Chemistry 1997, 45, 1920-1930); 136-95-8 (Sigma-Aldrich, St. Louis, MO, catalog no. 108812); 4570-41-6; 40926-66-7 (see Kalcheva et al, Zeitschrift fuer Chemie 1981, 21, 219-220); 114498-55-4 (Chemstep, Carbon Blanc, France, catalog no. 17590); 118767-91-2 (Chemstep, Carbon Blanc, France, catalog no. 17511), 4592-62-5, 82357-92-4 (see Goerdeler and Horn, Ber. 1963, 96, 1551-60); 1820-80-0 (Sigma-Aldrich, St. Louis, MO, catalog no. 160644); 6826-24-0 (see Van Leusen et al, Journal of Organic Chemistry 1981, 46, 2069-72 and Tanimoto et al, Chemical & Pharmaceutical Bulletin 1984, 32, 1032-1039) and 33119-65-2 (see Crank, et al, Australian Journal of Chemistry 1985, 38, 447-458).

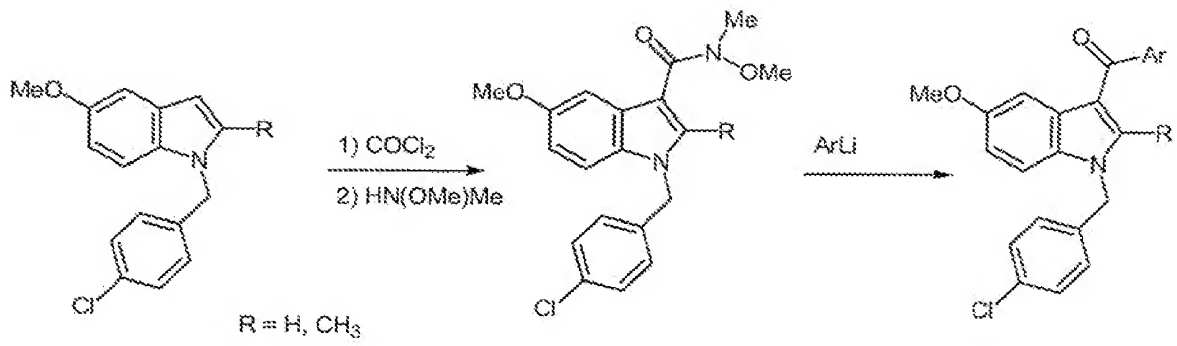
They also include those disclosed in Van Leusen et al. Journal of Organic Chemistry 1981, 46, 2069-2072 such as


,

,and

and

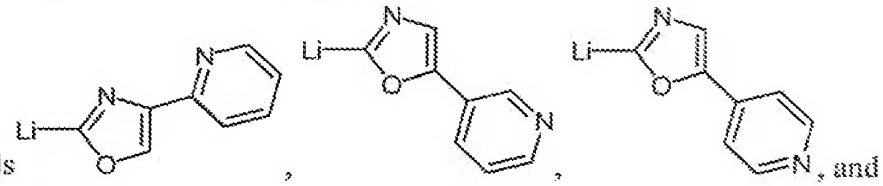
Herdeis and Gebhard Heterocycles 1986, 24, 1019-1024 such as


,

, and

.

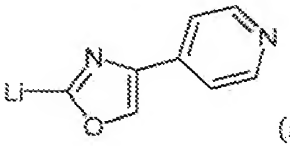
General method for the preparation of selected ketone derivatives.



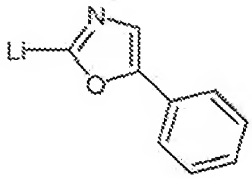
Starting materials for the preparation of ArLi reagents include: CA Registry Numbers 20662-89-9 (see Whitney et al. Journal of Organic Chemistry 1990, 55, 929-935); 133229-54-6 (see Whitney et al. Journal of Organic Chemistry 1991, 56, 3058-3063); 681135-55-7 (Chemstep, Carbon Blanc, France, catalog no. 15867); 681135-57-9 (Chemstep, Carbon Blanc, France, catalog no. 15866); 35299-74-2 (see Boger et al. Journal of Medicinal Chemistry 2005, 48, 1849-1856); 681135-59-1(Chemstep, Carbon Blanc, France, catalog no. 15865); 1006-68-4 (Ryan Scientific, Inc., Isle of Palms, SC, catalog no. SEW 04470); 70380-73-3 (Ryan Scientific, Inc., Isle of Palms, SC, catalog no. SEW 00968); 70380-74-4 (Ryan Scientific, Inc., Isle of Palms, SC, catalog no. SEW 00967); 70380-75-5 (Ryan Scientific, Inc., Isle of Palms, SC, catalog no. SEW 00891); 121855-80-9 (see Davies et al. Journal of the Chemical Society, Perkin Transactions 1: Organic and Bio-Organic Chemistry 1989, 837-838);



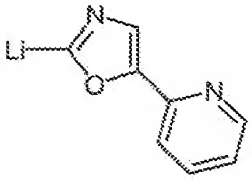
as well as compounds , , , and



(see Boger et al. Journal of Medicinal Chemistry 2005, 48, 1849-1856);

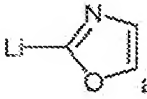


(see Crowe et al. Tetrahedron 1995, 51, 8889-8900);

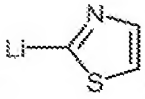


(see Dondoni et al. Journal of Organic Chemistry 1987, 52, 3413-3420),

and

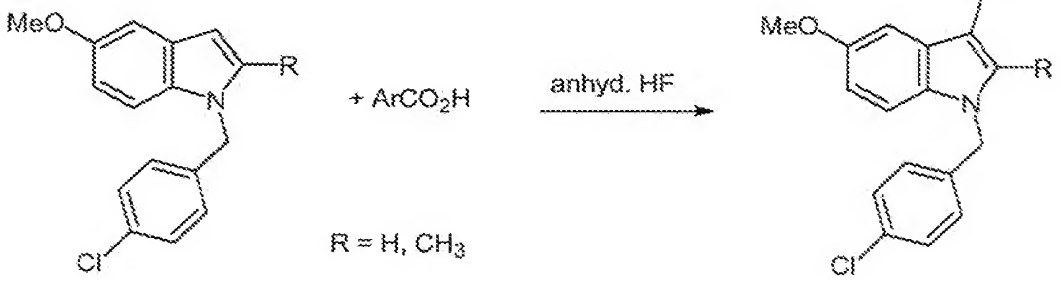


and



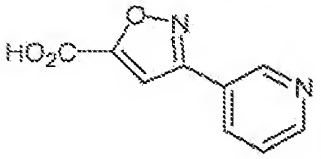
(see Subramanyam et al. Tetrahedron Letters 2002, 43, 6313-6315.

General method for the preparation of selected ketone derivatives by Friedel-Crafts acylation of an indole.



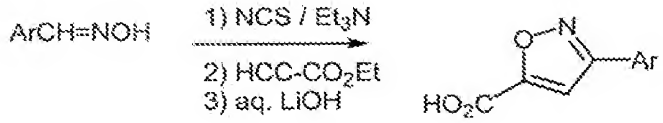
The following compounds (Ar) can be used for the preparation of carboxylic acids for acylation of indole in the 3-position and are either commercially available or can be derived from the literature methods as described: CA Registry Nos. 16042-25-4 (Maybridge plc, Tintagel, Cornwall, United Kingdom, catalog no. CC 08901); 59020-44-

9 (J & W PharmLab LLC, Morrisville, PA, catalog no. 90-0085); 75390-44-2 (ACB Blocks Ltd, Moscow Russia, catalog no. THA-0001); 59020-45-0 (Anichem LLC, Monmouth Junction, NJ, catalog no. S10224);59020-46-1 (see PCT publication WO2002014311); 59020-47-2 (Anichem LLC, Monmouth Junction, NJ, catalog no. S10225); 862494-59-5 (see PCT publication WO2005074645); 721927-07-7 (see PCT publication WO2004058728); 794500-94-0 and 723733-05-9 (see PCT publication WO2004058728); 19163-24-7 (ASDI Inc, Newark, DE, catalog no. 500022101); 119082-97-2 ((ASDI Inc, Newark, DE, catalog no. 500021267); 35299-74-2 (see Kauffmann et al. Angewandte Chemie, International Edition in English 1971, 10, 741-3); 278803-20-6 and 216867-32-2 (AKos Consulting and Solutions GmbH, Basel Switzerland, catalog nos. BBV-00006978 and BBV-00011817); 527-72-0; 21169-71-1 and 14442-12-7 (Sigma-Aldrich, St. Louis, MO, catalog nos. 88990, 636258, and 633690); 499770-97-7; 10271-85-9 (AstaTech, Inc., Bristol, PA, catalog no. 62856); 4576-90-3 (see Holland et al. Journal of the Chemical Society 1965, 7277-7282); 3209-71-0 (ChemPacific USA Sales Marketing and Research Center, Baltimore, MD, catalog no. 37117); 716362-11-7 and 716362-05-9 (Ambinter, Paris France, catalog nos. CIZ-0020 and CIZ-0022), and

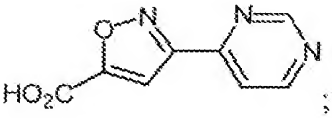


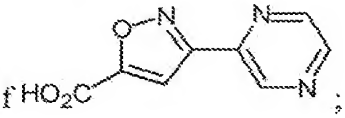
(see PCT publication WO2004054974).

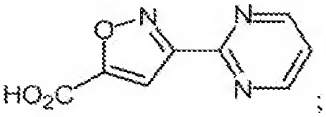
General method for the construction of 3-aryl-5-carboxy isoxazoles by dipolar cycloaddition of nitrile oxides (generated from oximes with N-chlorosuccinimide and triethylamine) with ethyl propiolate.

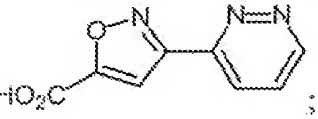


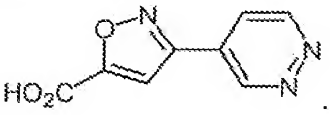
Using this methodology,

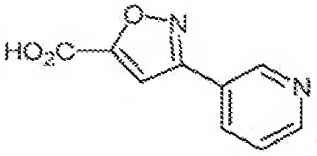
CA registry no. 1073-65-0 (Chemstep, Carbon Blanc, France, catalog no. 20123) can be used for the preparation of  ;

CA registry no. 1193-98-2 (see Jose et al. Synthetic Communications 2000, 30, 1509-1514) can be used for the preparation of  ;

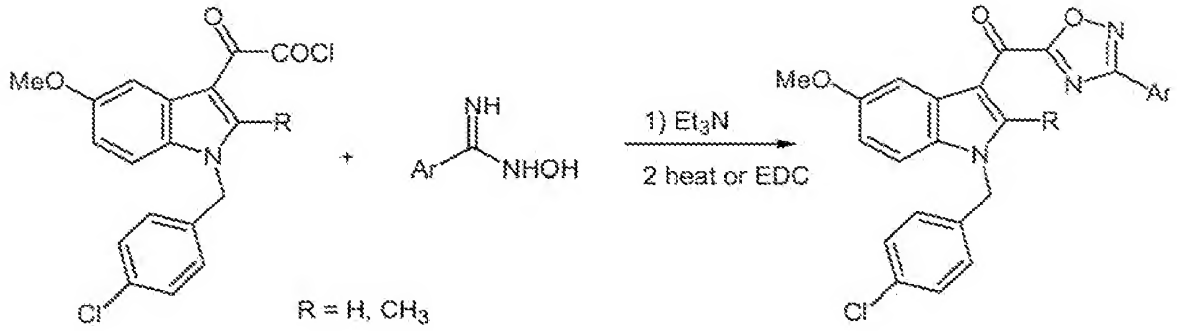
CA registry no. 83959-46-0 (Chemstep, Carbon Blanc, France, catalog no. 18812) can be used for the preparation of  ;

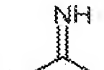
CA registry no. 52348-44-4 (see EP patent publication EP194746A2) can be used for the preparation of  ;

and CA registry no. 50901-50-3 (Chemstep, Carbon Blanc, France, catalog no. 5053) can be used for the preparation of  .

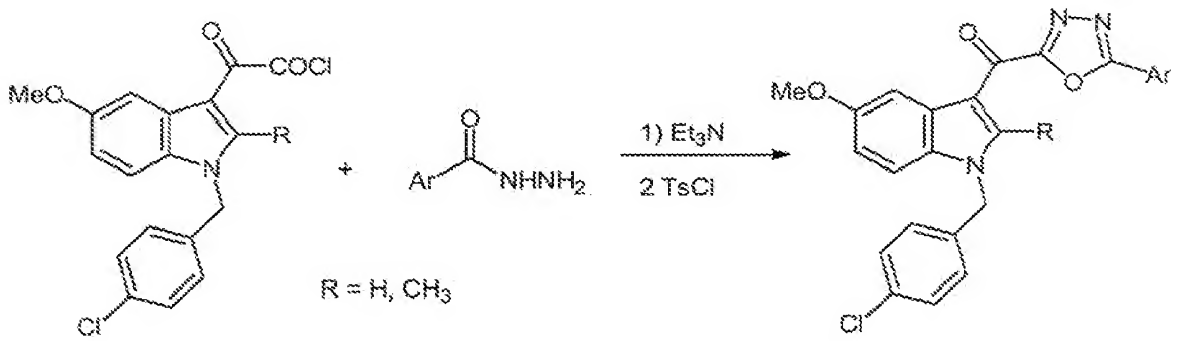
 is prepared as described in US3957805.

General method for the preparation of 1,2,4-oxadiazole containing ketone derivatives.



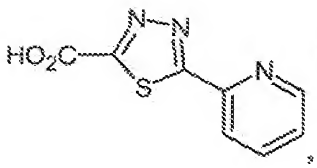
The following is a list of commercially available or literature references for the preparation of carboximides  : CA Registry Nos. 613-92-3, 1772-01-6, 1594-58-7, and 1594-57-6 (Sigma-Aldrich, St Louis, MO, catalog nos. S778176, 542792, 542814, and 542806); 51285-11-1 and 90993-49-0 (Chemstep, Carbon Blanc, France, catalog nos. 19221 and 18704); 90993-48-9 (see Easmon et al. Journal of Medicinal Chemistry 1992, 35, 3288-3296 for the preparation of 3-cyanopyridazine and Robba Ann. Chim. (Paris) 1960, 380, 414 for the preparation of 3-pyridazinecarboxamidoxime); 51285-05-3 (Oakwood Products, Inc, West Columbia, SC, catalog no. 017309), and 39123-45-0 (see US3705157).

General method for the preparation of 1,3,4-oxadiazole containing ketone derivatives.



The following is a list of commercially available or literature references for obtaining acylhydrazides.

CA Registry No. 89463-74-1; 87362-28-5; 56932-26-4 (Chemstep, Carbon Blanc, France, catalog no. 29632, 18794, 29637); 39513-54-7; 768-05-8 (J & W PharmLab LLC, Morrisville, PA, catalog no. 70-0046, 65-0113); 103028-60-0 (see Ohta et al. Nippon Kagaku Zasshi 1958, 79, 1452-1454); 13363-69-4 (see Beringer et al. Helvetica Chimica Acta 1966, 49, 2466-2469), 52938-97-3 (TCI America, Portland, OR, catalog no. P1394); 862089-25-6, 56632-46-3, and 216867-35-5 (AKos Consulting and Solutions GmbH, Basel, Switzerland, catalog nos. BBV-00011847, BBV-00011848, and BBV-00011849); and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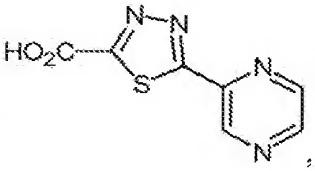
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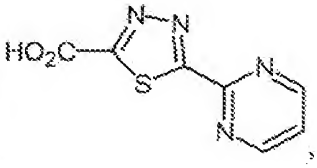
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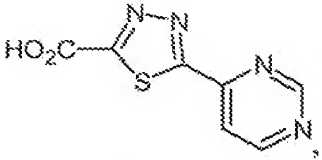
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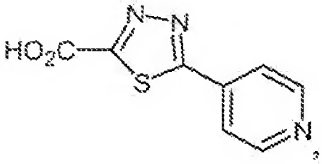
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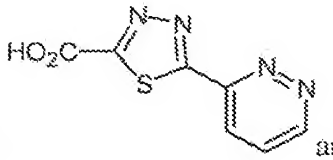
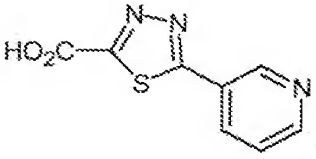
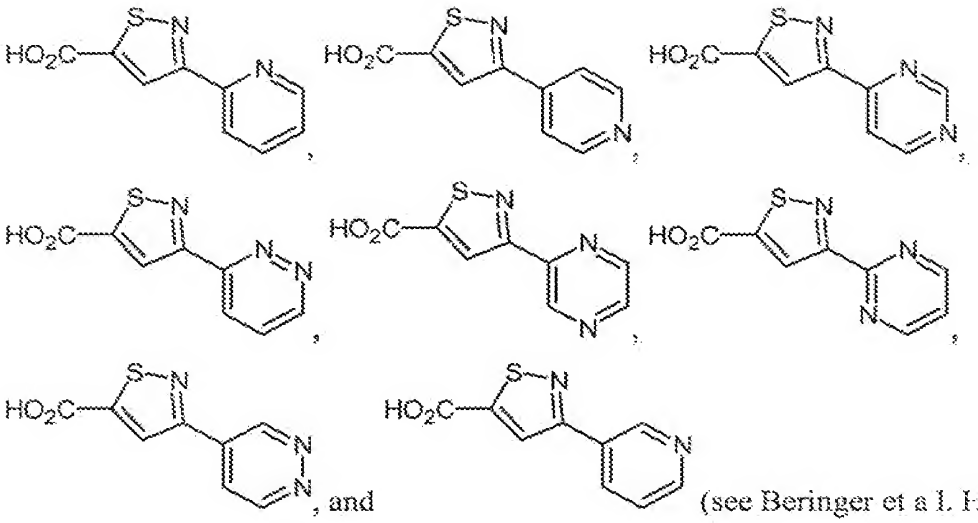
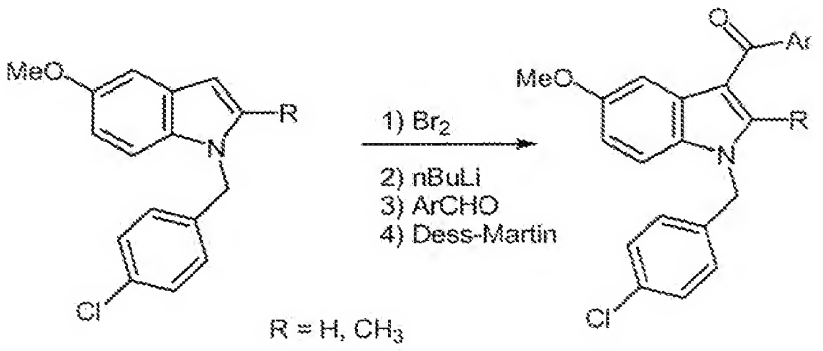

and

(see WO9854164);

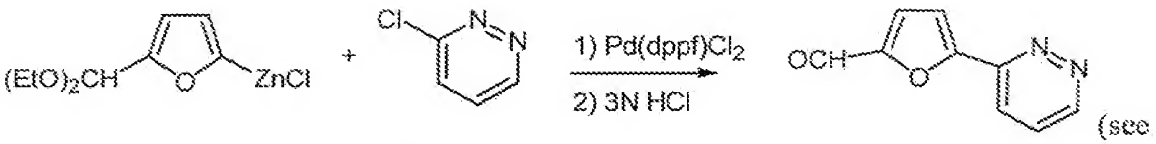


, and (see Beringer et a l. Helvetica Chimica Acta 1966, 49, 2466-2469).

General method for the preparation of indole ketones by reaction of 3-lithio-indole with an aldehyde followed by oxidation with the Dess-Martin reagent.

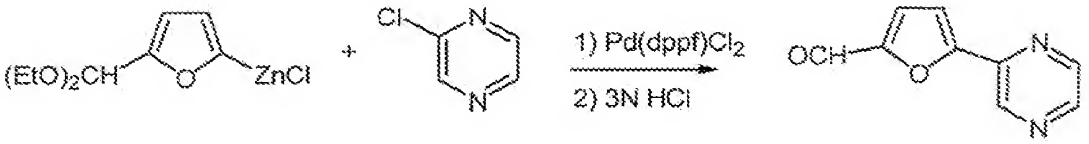


The following is a list of commercially available or literature references for obtaining the requisite aldehydes (ArCHO in above reaction), CA registry numbers: 342601-31-4 (see PCT publication WO2001038332); 1120-95-2 (see Wernuth et al. Journal of Medicinal Chemistry 1987,30, 239-249); CA registry number 1120-95-2 can be used to synthesize and aldehyde in the following reaction



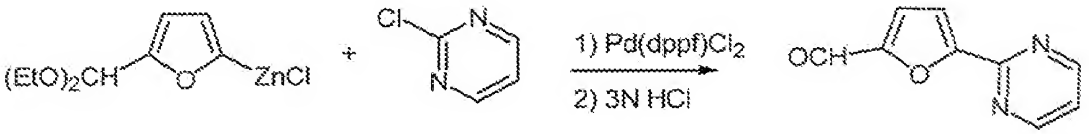
(see Gauthier et al Org. Lett., 2002, 4, 375 –378);

CA Registry no. 342603-67-2 which can be derived from the following reaction:



(see Gauthier et al. Org. Lett., 2002, 4, 375 -378.);

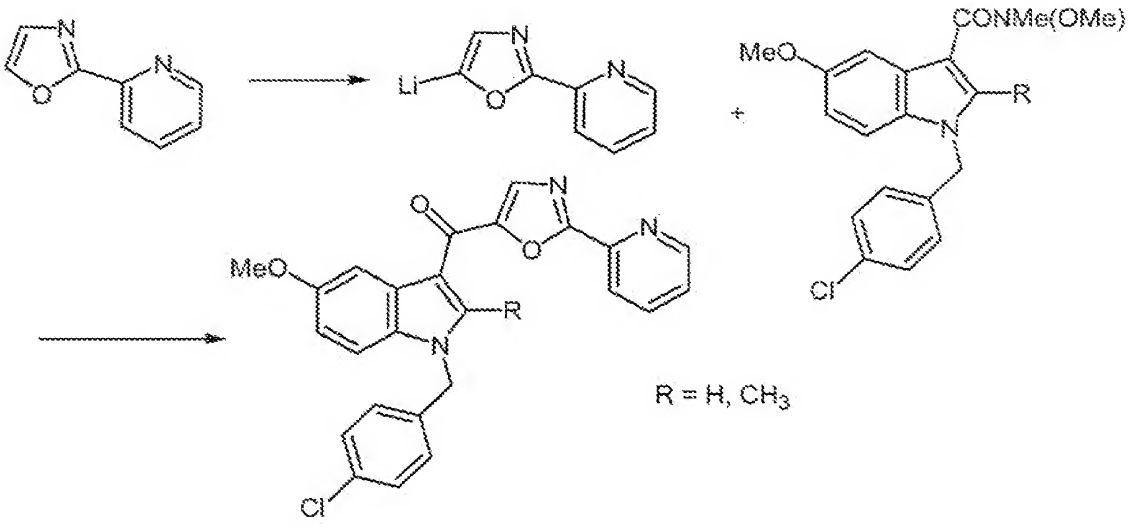
CA Registry no. 545445-71-4 which can be derived from the following reaction:



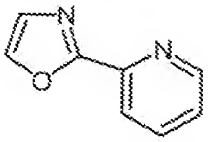
(see Gauthier et al. Org. Lett., 2002, 4, 375 -378.);

CA Registry no. 342601-17-6 (see WO2001038332); and CA Registry no. 106833-79-8 (see WO2002016355);

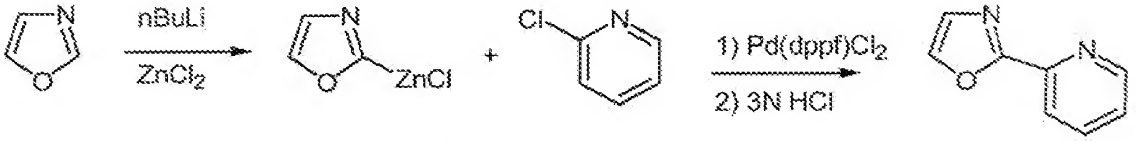
General method for the preparation of ketone derivatives by reaction of an organometallic reagent with the indole Weinreb amide.



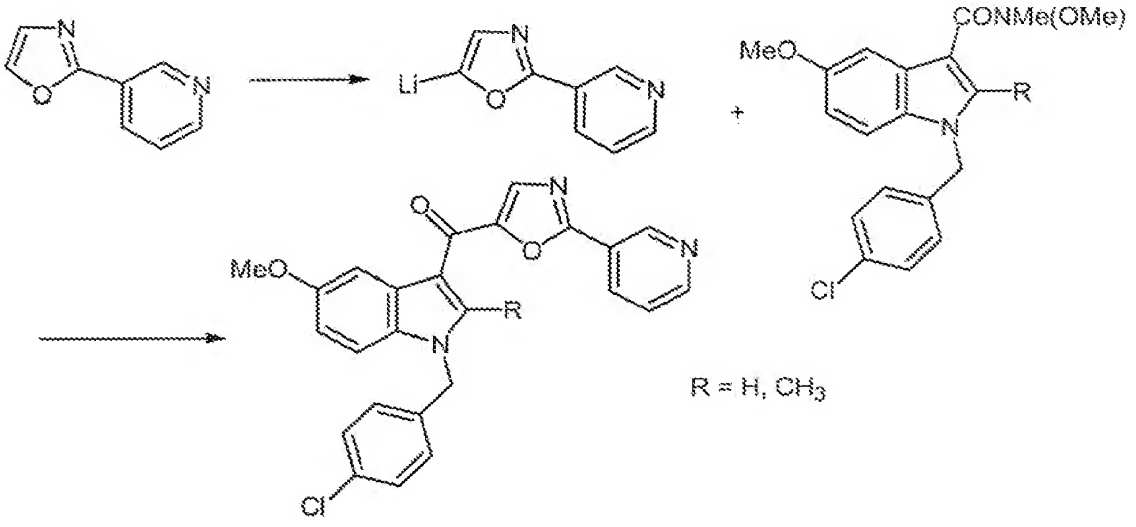
See Boger et al. Journal of Medicinal Chemistry 2005, 48, 1849-1856. Starting material,

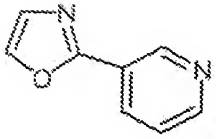


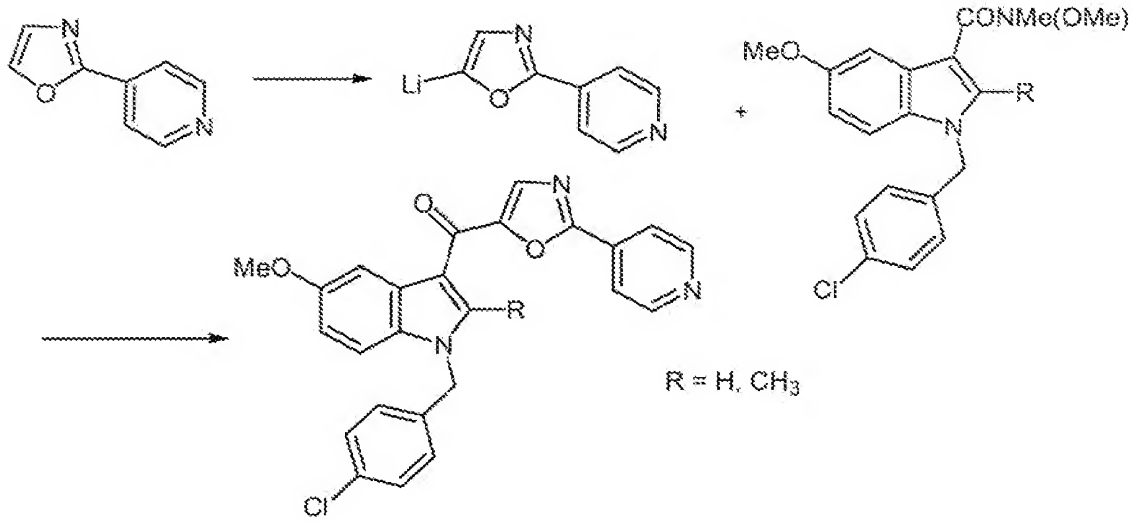
(CA Registry no. 5998-89-0) can be obtained by processes described in Dadkhah and Prijs 1962, 45, 375-381 or using the following reaction:

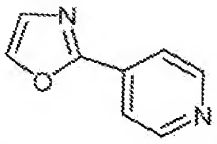


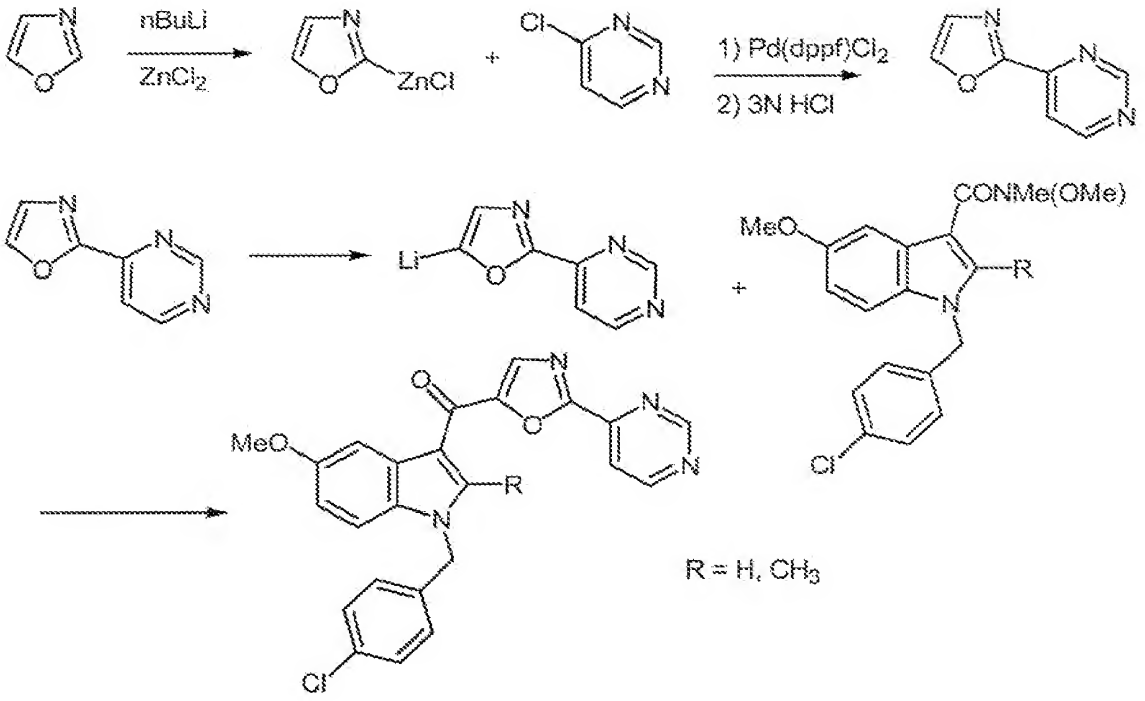
which is described in Gauthier et al. Org. Lett., 2002, 4, 375 -378.



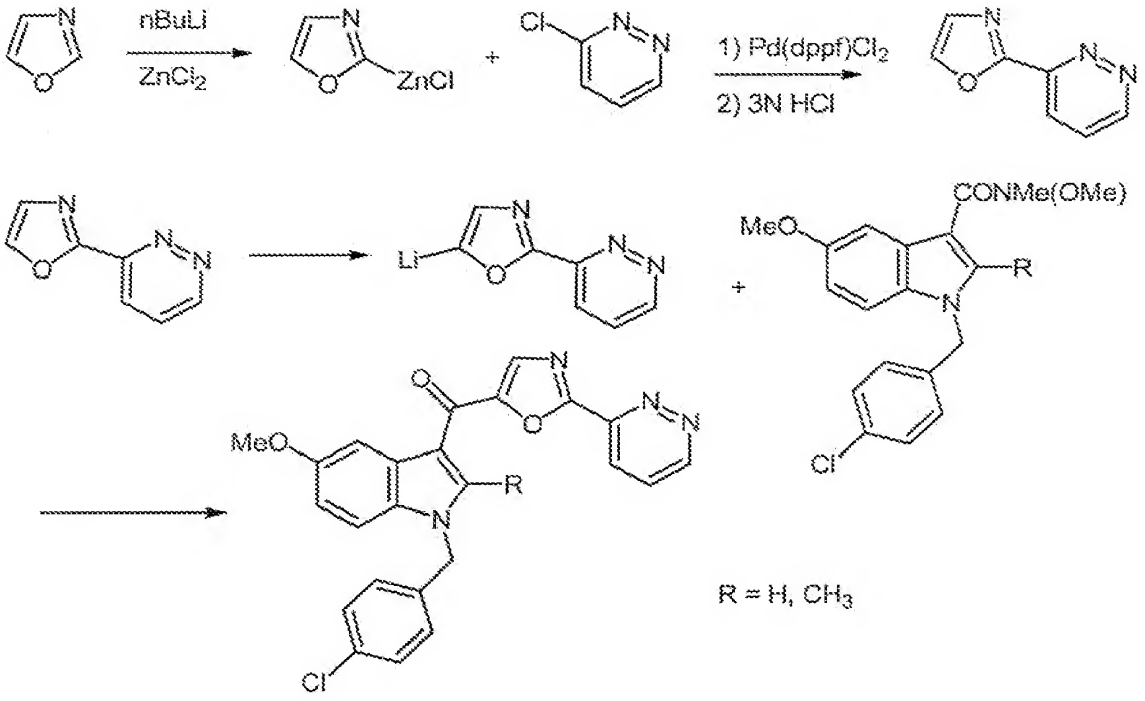
See Boger et al. Journal of Medicinal Chemistry 2005, 48, 1849-1856. For starting material,  (CA Registry no. 5998-85-6) see Dadkhah and Prijs 1962, 45, 375-381.



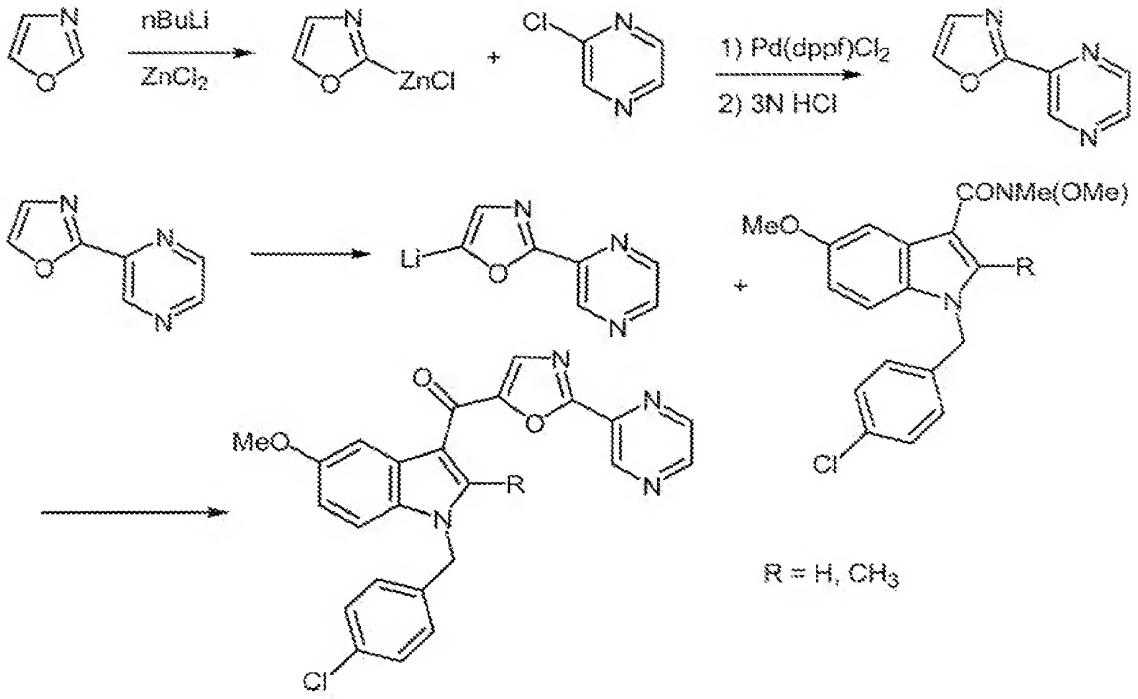
Starting material  (CA Registry no. 5998-92-5) is available from the Florida Center for Heterocyclic Compounds (Gainesville, FL, catalog no. 1265).



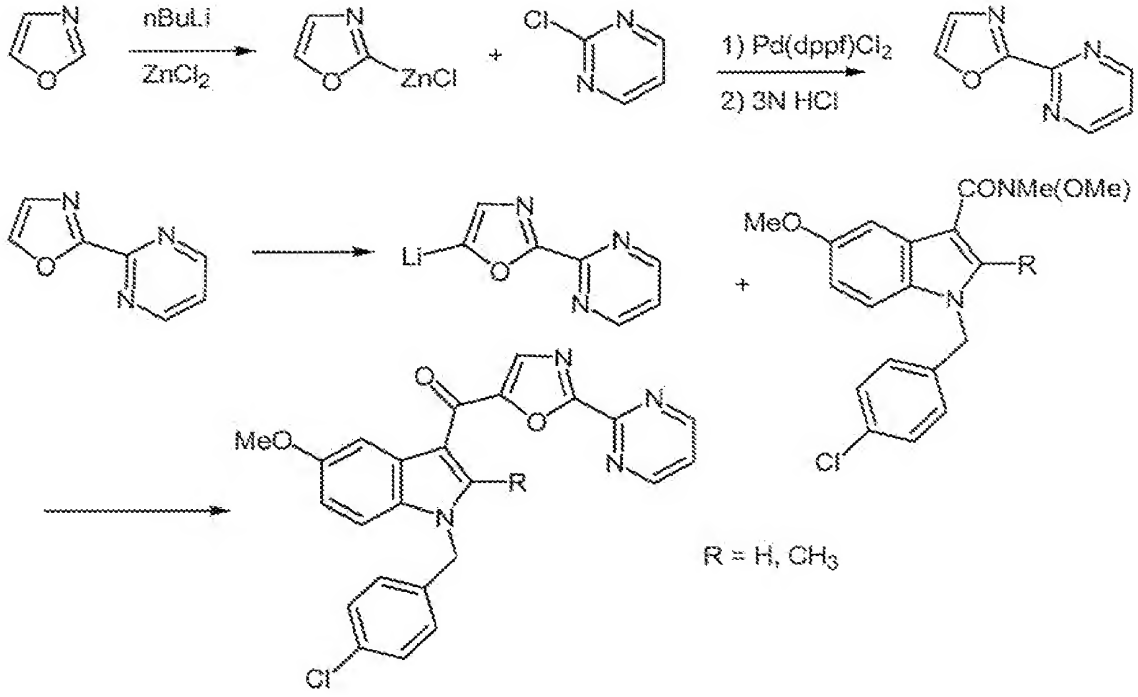
See Gauthier et al Org. Lett., 2002, 4, 375 -378 and Boger et al. Journal of Medicinal Chemistry 2005, 48, 1849-1856.



See Gauthier et al Org. Lett., 2002, 4, 375 -378 and Boger et al. Journal of Medicinal Chemistry 2005, 48, 1849-1856.



See Gauthier et al Org. Lett., 2002, 4, 375 -378 and Boger et al. Journal of Medicinal Chemistry 2005, 48, 1849-1856.



See Gauthier et al Org. Lett., 2002, 4, 375 -378 and Boger et al. Journal of Medicinal Chemistry 2005, 48, 1849-1856.

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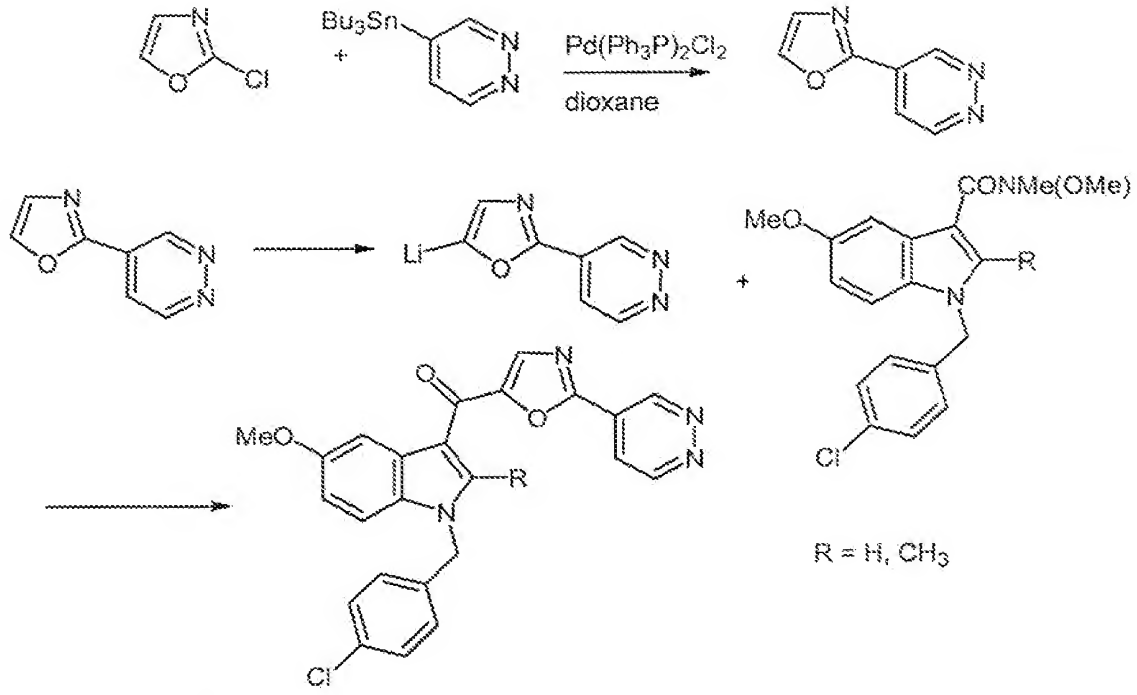
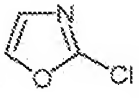
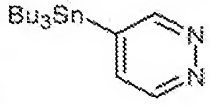
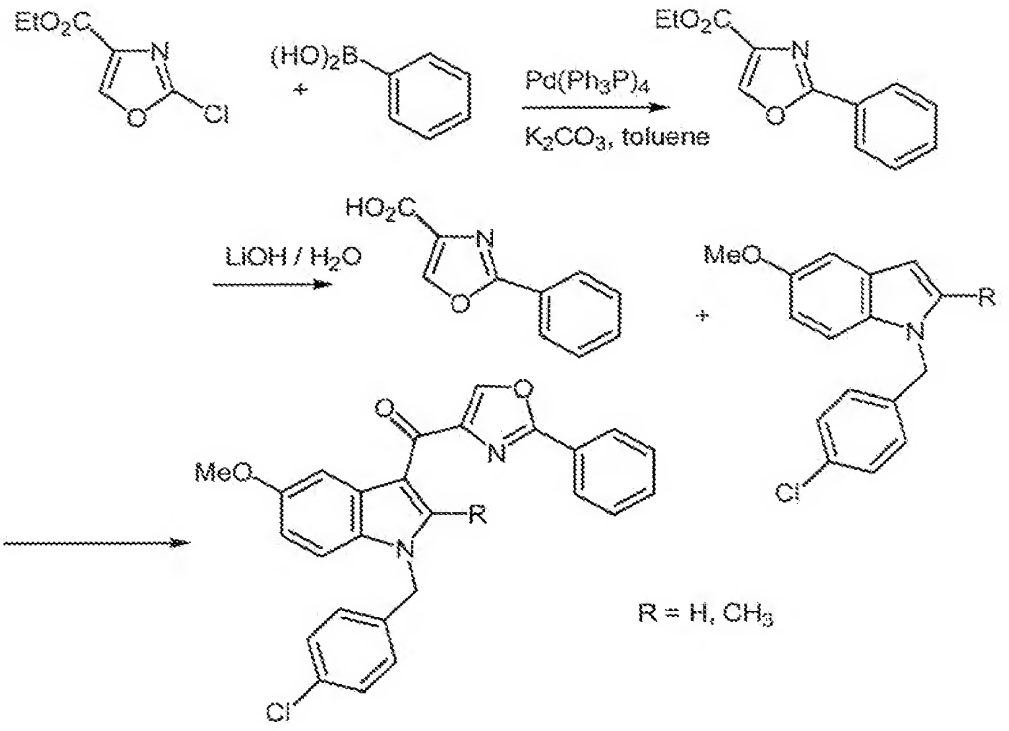
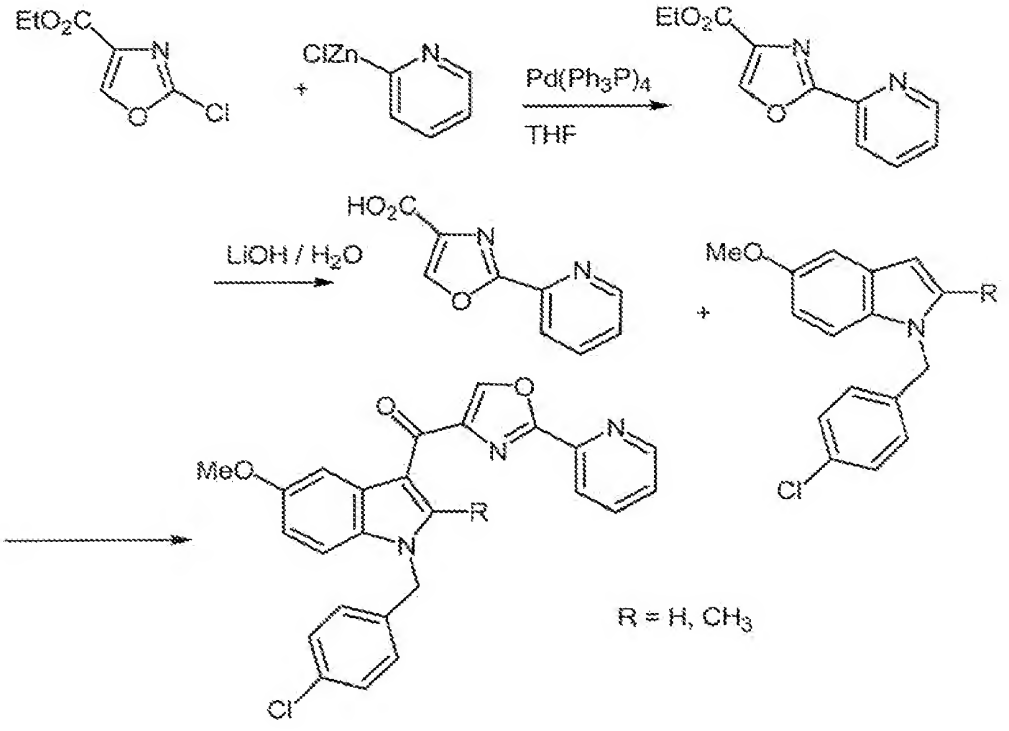


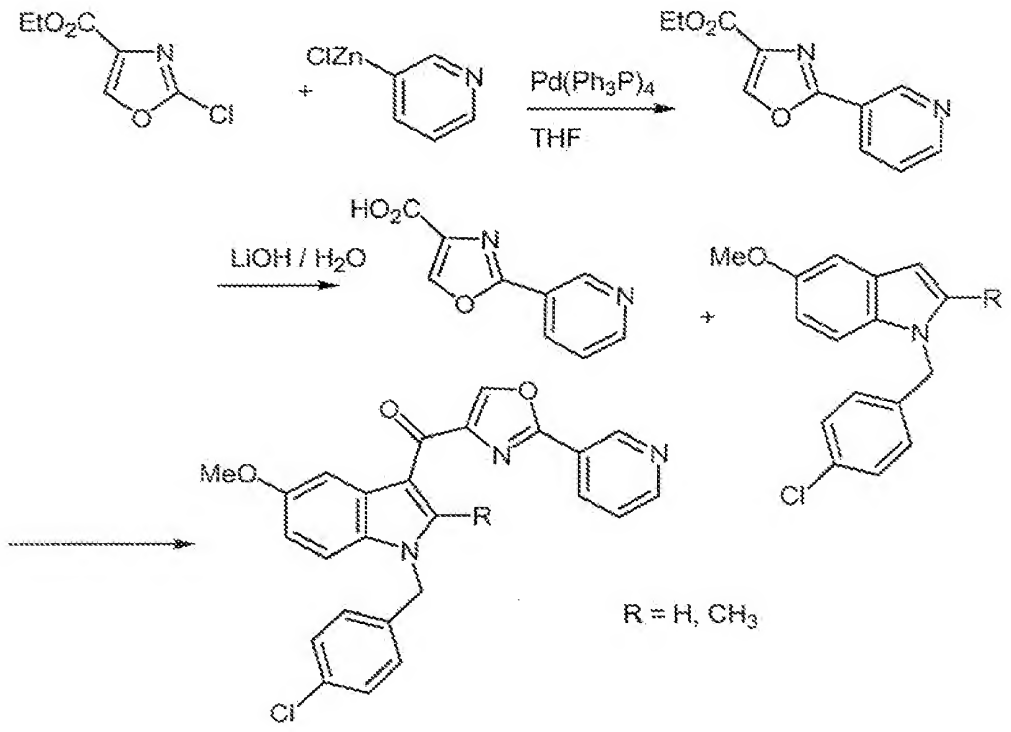
For reagent  (CA registry no. 95458-77-8) see Haviv et al. Journal of Medicinal Chemistry 1988, 31, 1719-1728. For reagent  see Heldmann et al. Tetrahedron Letters 1997, 38, 5791-5794



See Hodgetts et al. Org. Lett., 2002, 4, 2905-2907.



See Hodgetts et al. Org. Lett., 2002, 4, 2905-2907.



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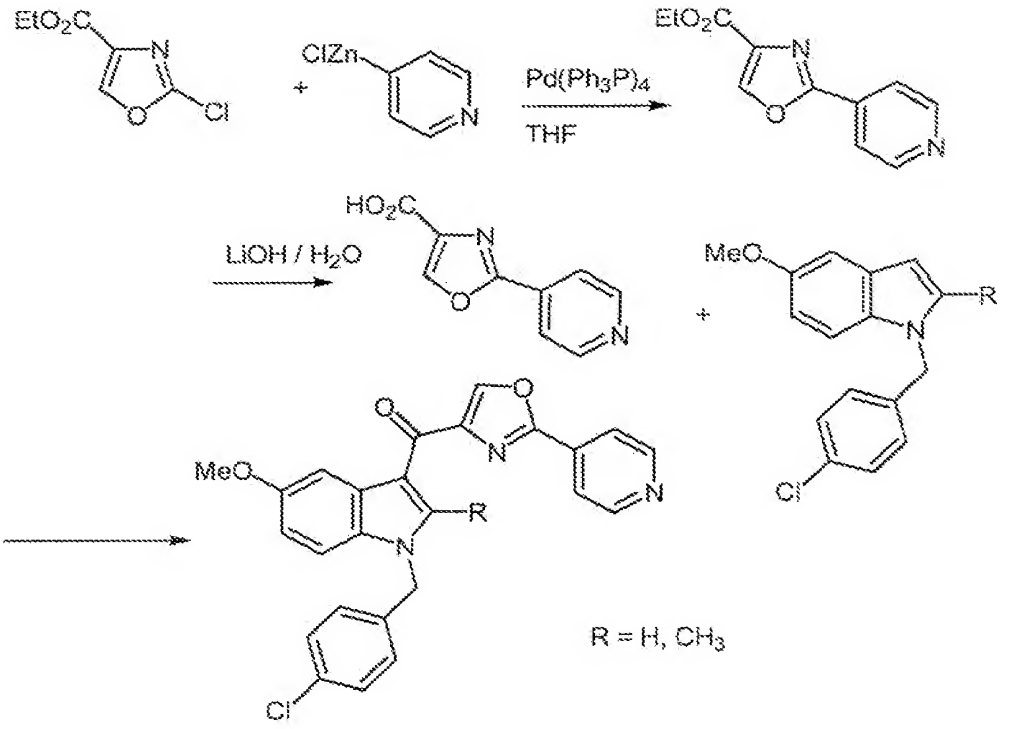

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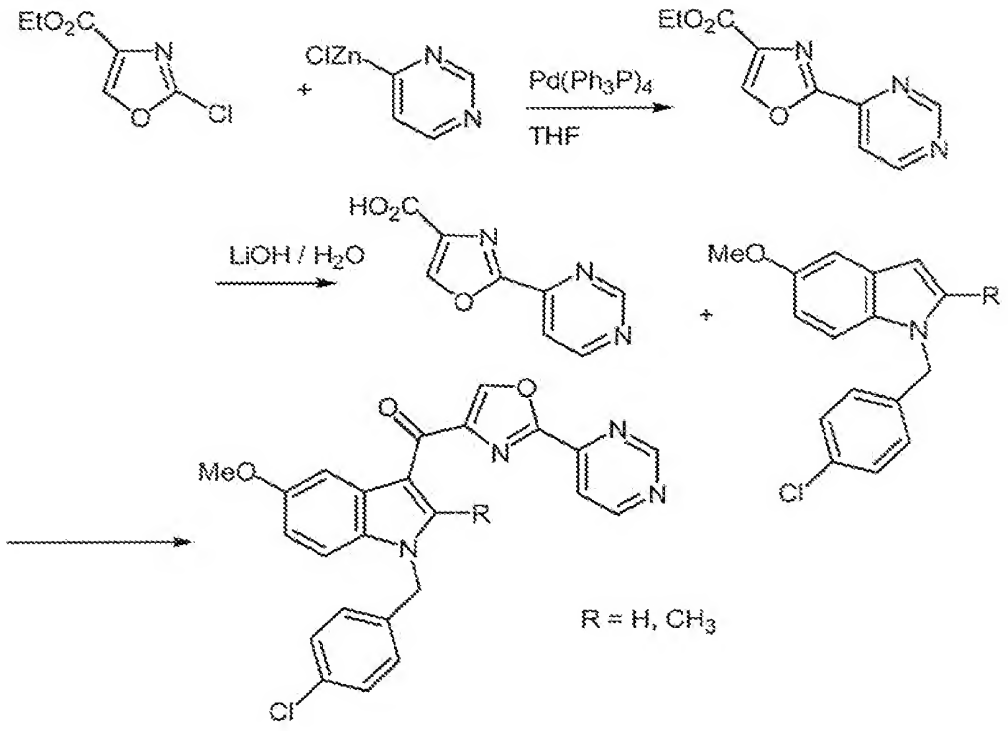

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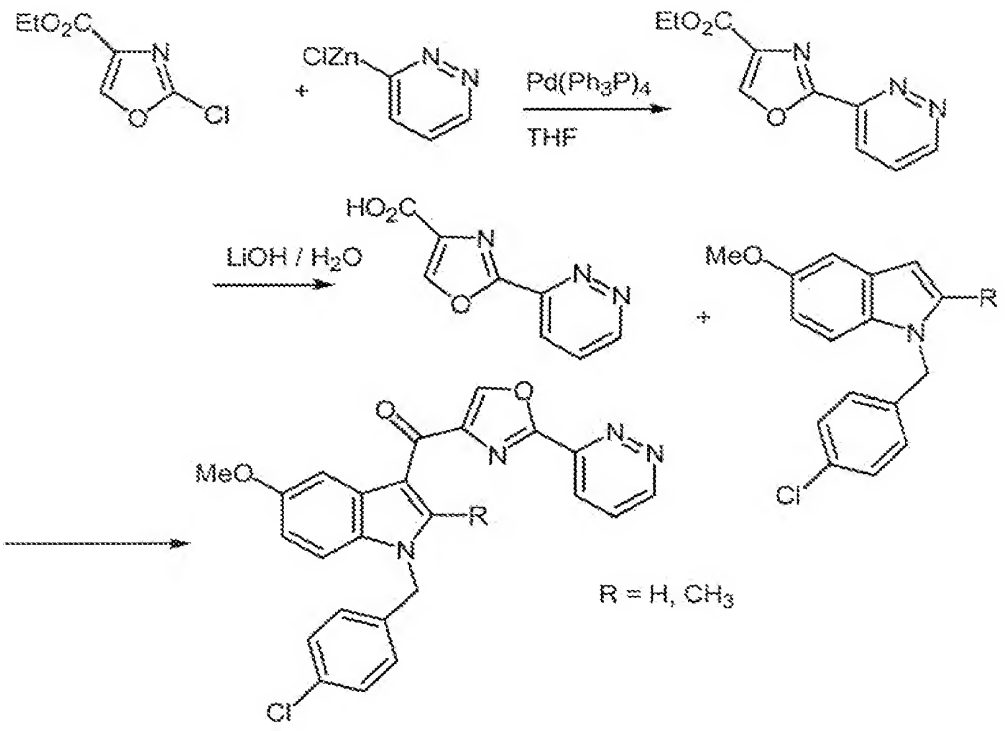

See Hodgetts et al. Org. Lett., 2002, 4, 2905-2907.

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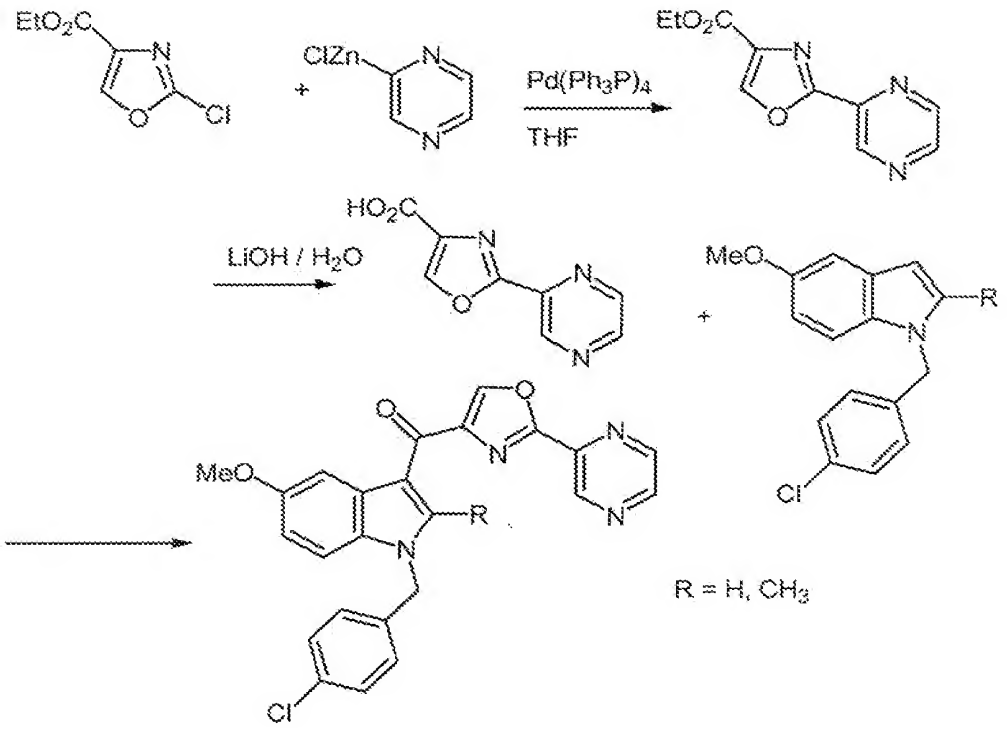
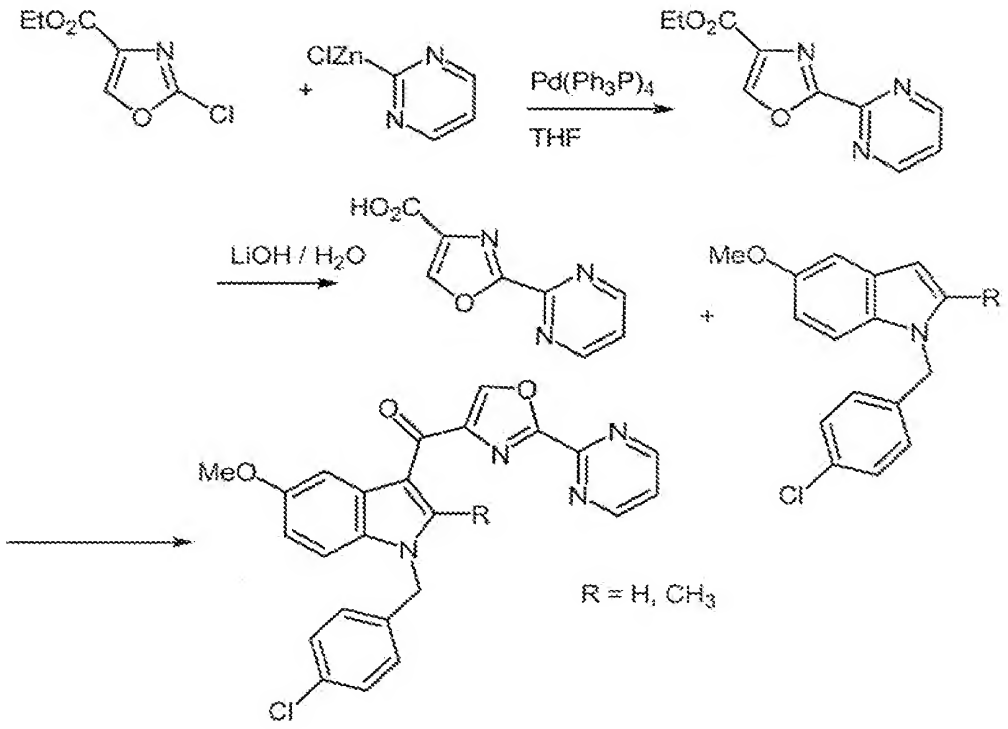


See Hodgetts et al. Org. Lett., 2002, 4, 2905-2907.



See Hodgetis et al. Org. Lett., 2002, 4, 2905-2907.

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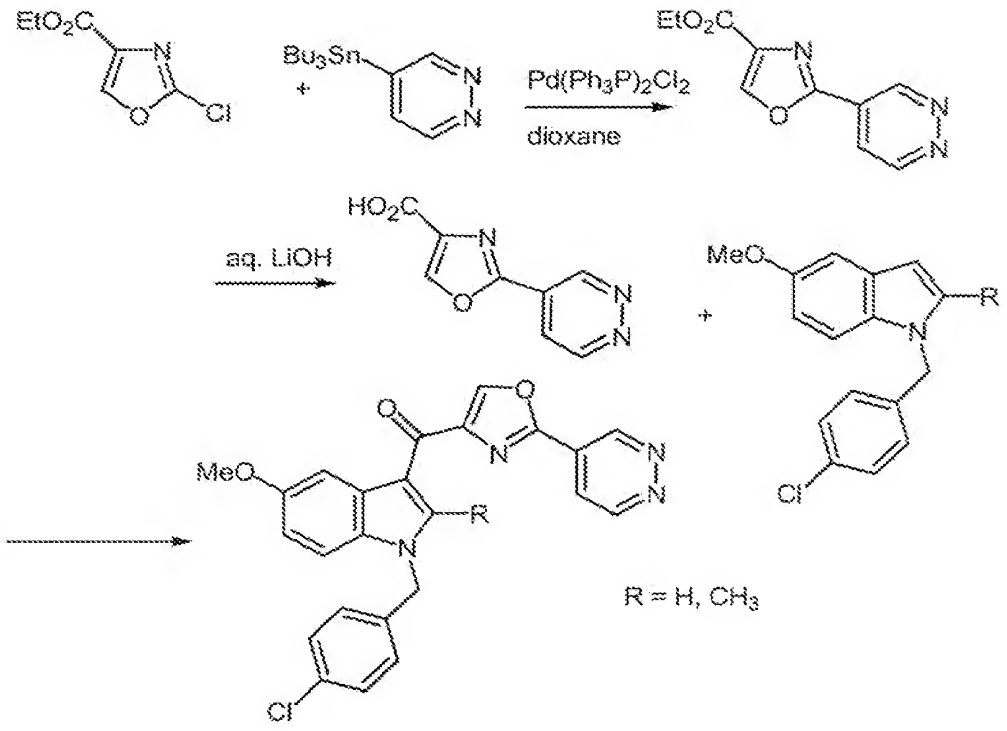

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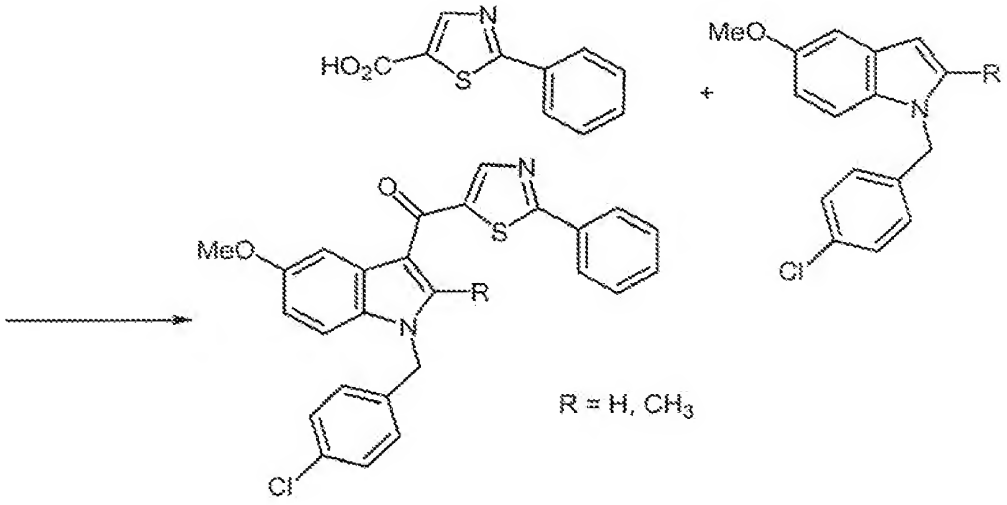
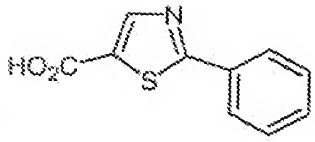
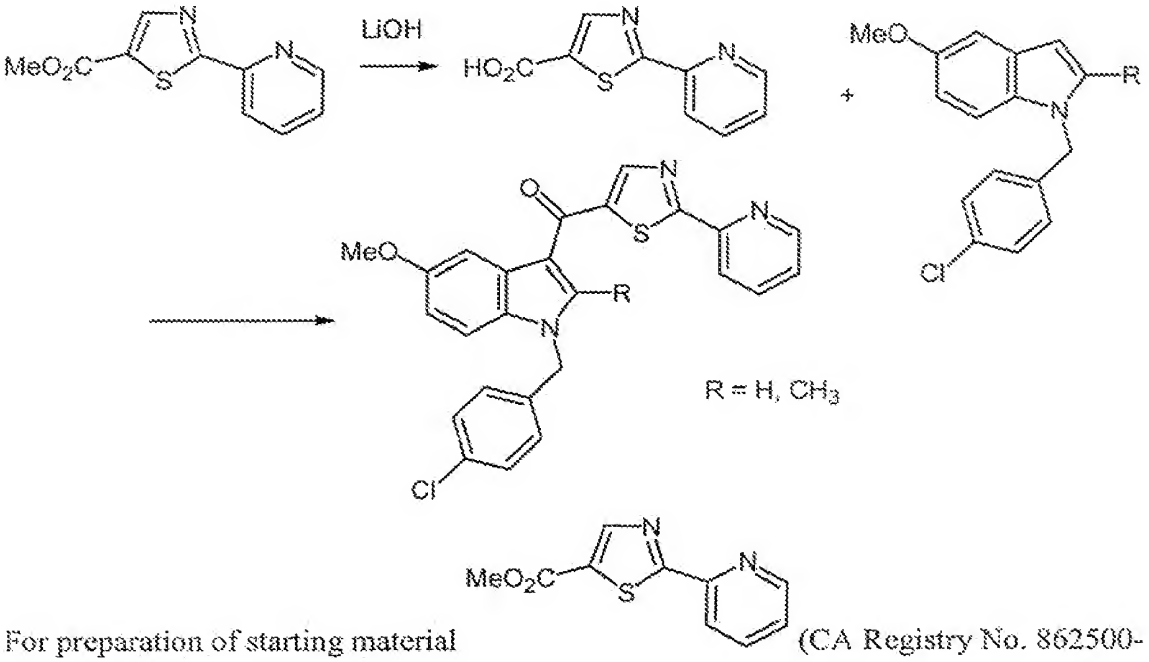
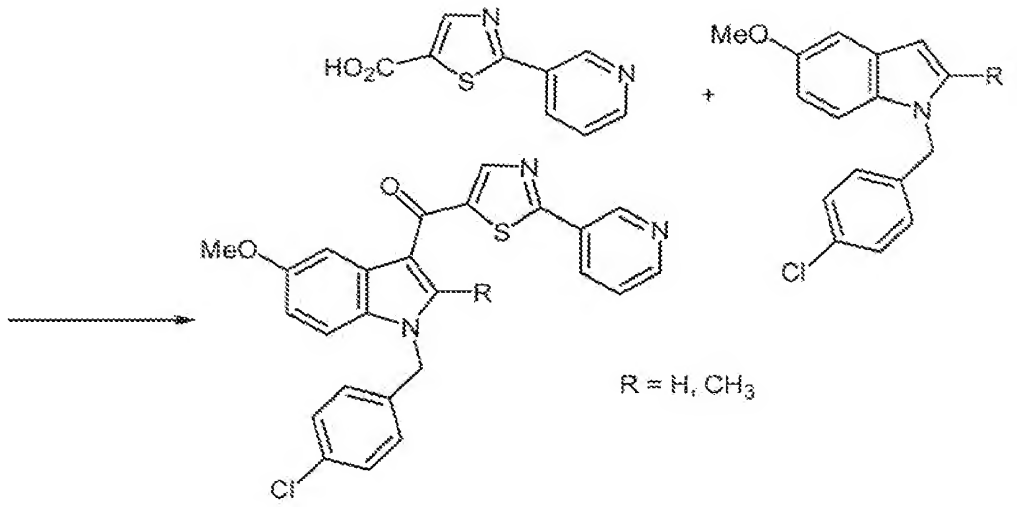


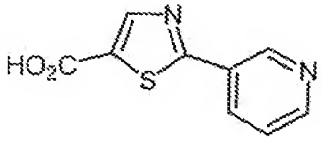


Reagent (CA Registry no. 10058-38-5) is available from MicroChemistry Ltd. (Moscow, Russia, catalog no. thiazole 750)

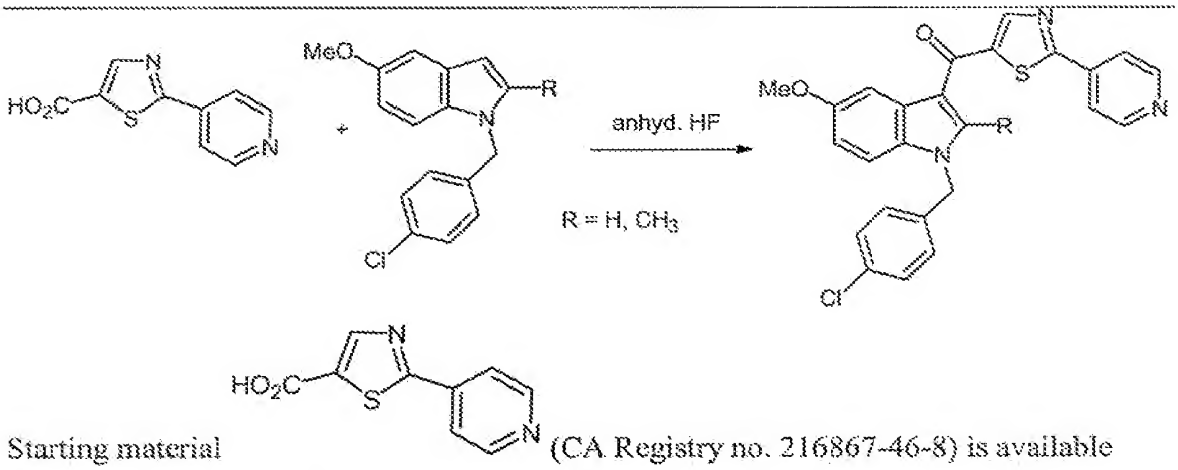


For preparation of starting material (CA Registry No. 862500-42-3) see WO2005075469.

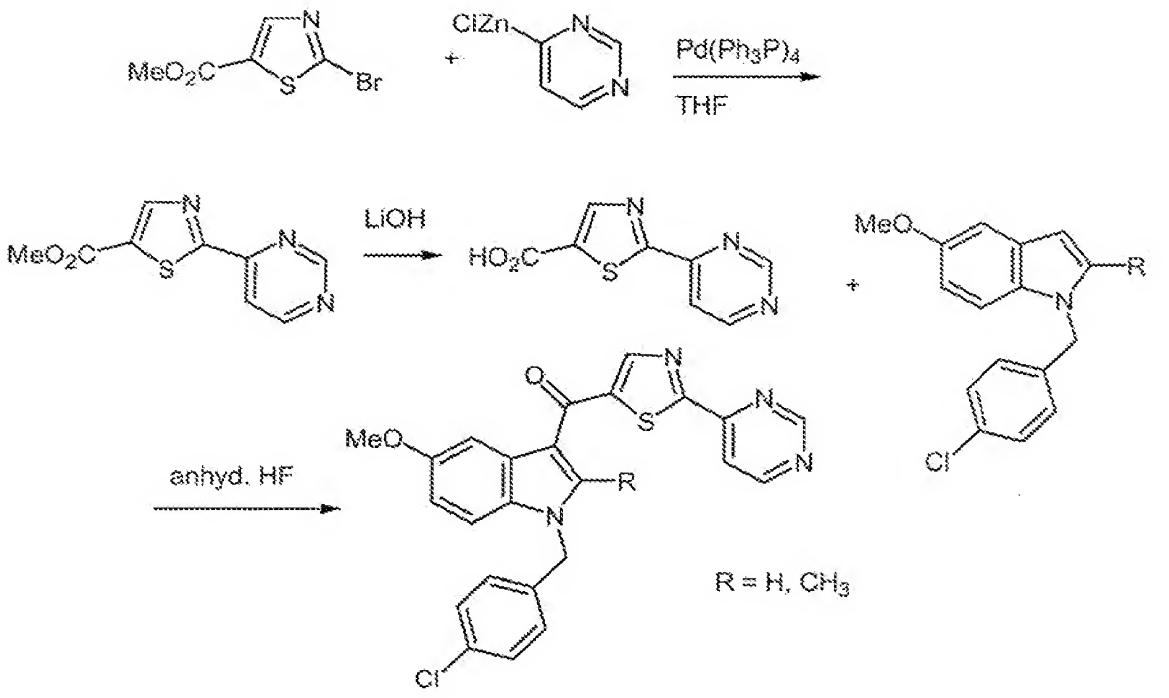


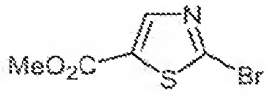


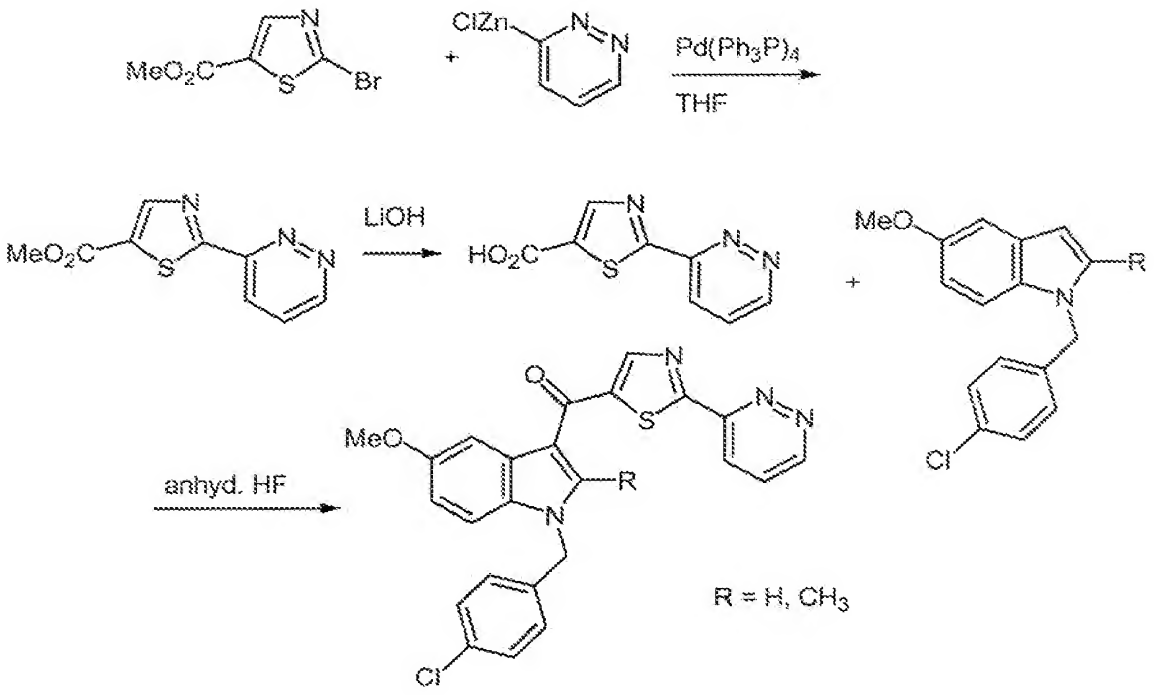
Starting Material (CA Registry no. 248275-42-5) is available from Anichem LLC (Monmouth Junction, NJ, catalog no. S10219)



Starting material (CA Registry no. 216867-46-8) is available from Anichem LLC (Monmouth Junction, NJ, catalog no. S10218)



See Hodgetts et al. Org. Lett. 2002, 4, 1363-1365. Starting material  (CA Registry no. 54045-74-8) is available from Combi-Blocks, LLC (San Diego, CA, HI-1117)



See Hodgetts et al. Org. Lett. 2002, 4, 1363-1365.

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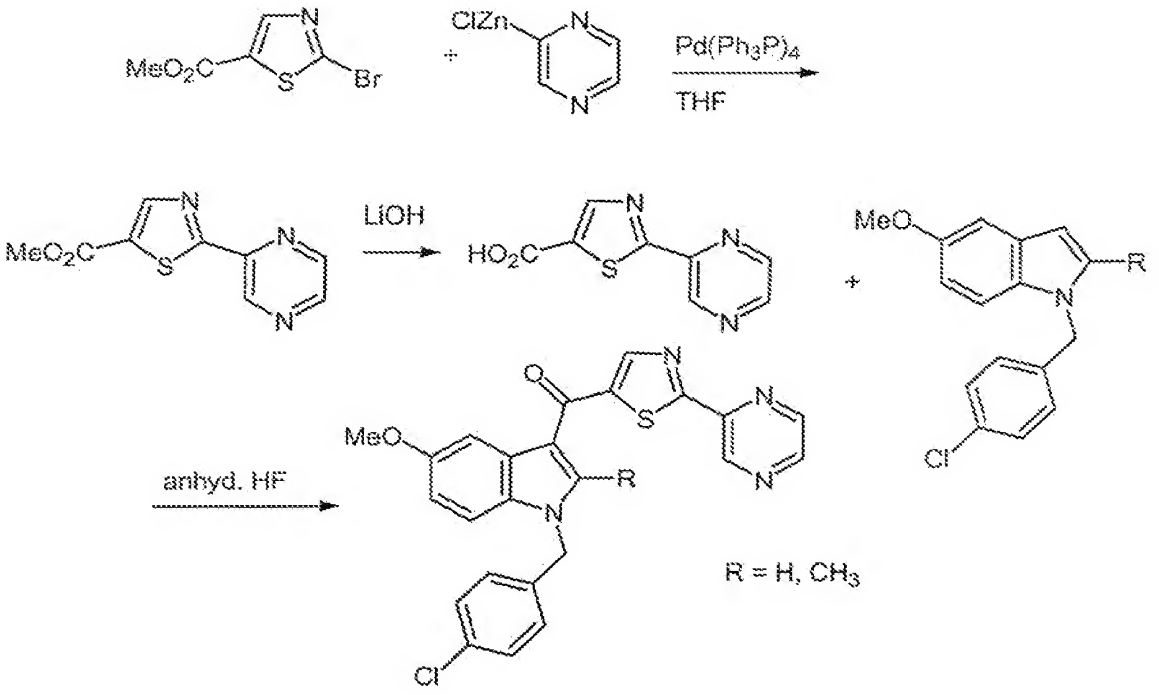

See Hodgetts et al. Org. Lett. 2002, 4, 1363-1365.

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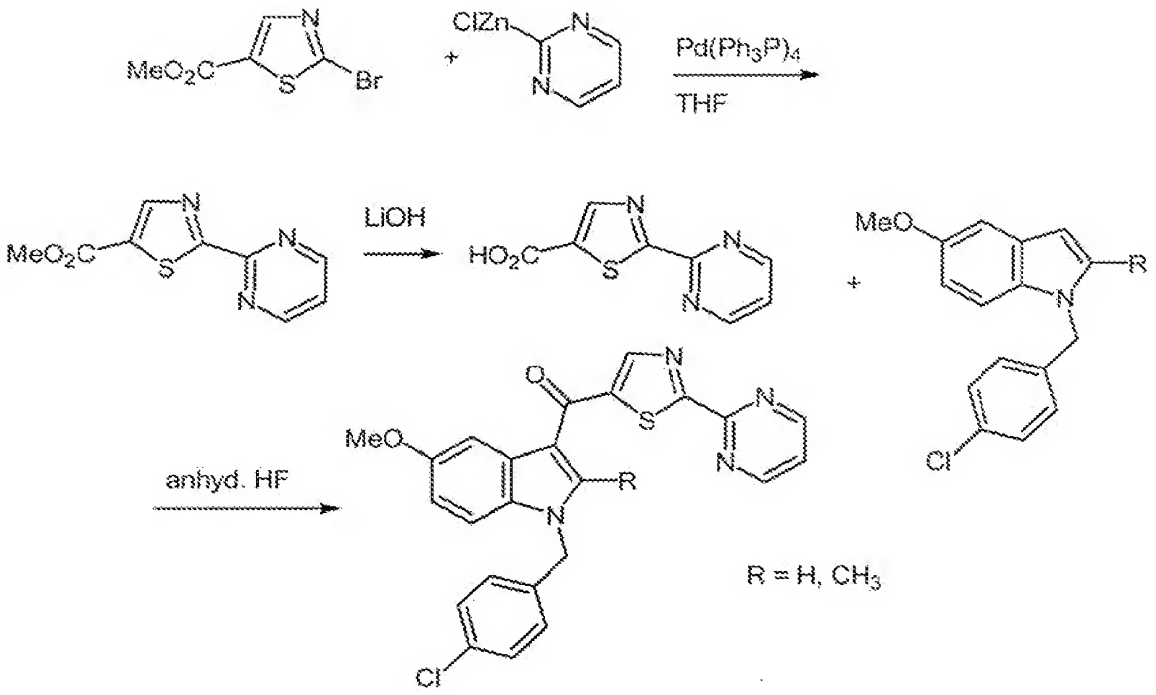

See Hodgetts et al. Org. Lett. 2002, 4, 1363-1365.

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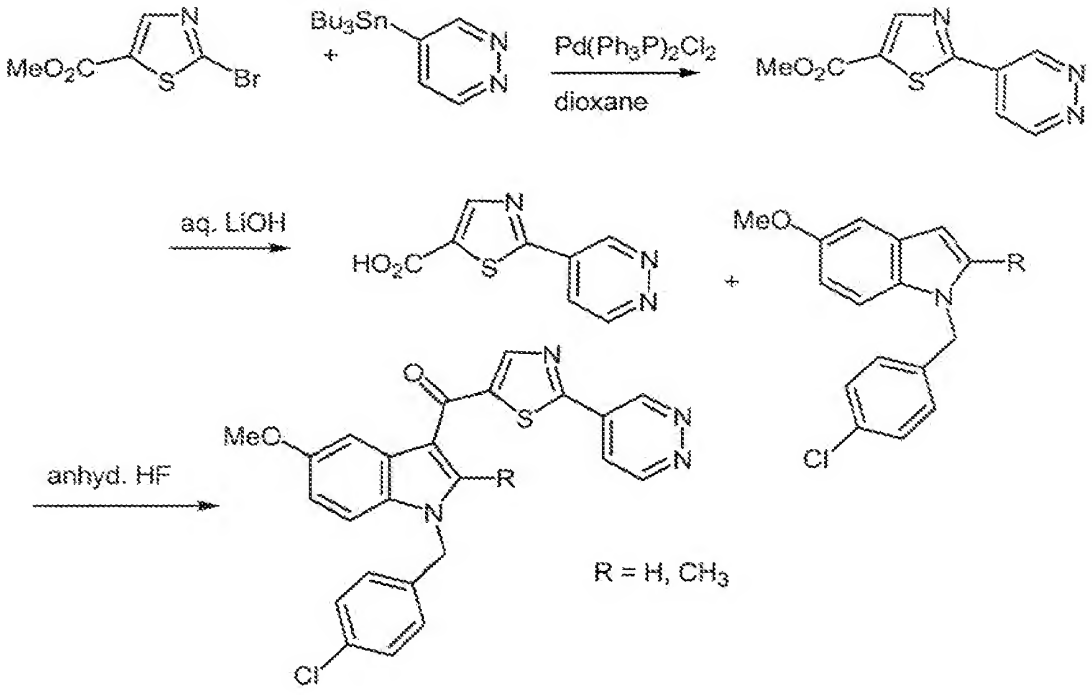

See Hodgetts et al. Org. Lett. 2002, 4, 1363-1365.

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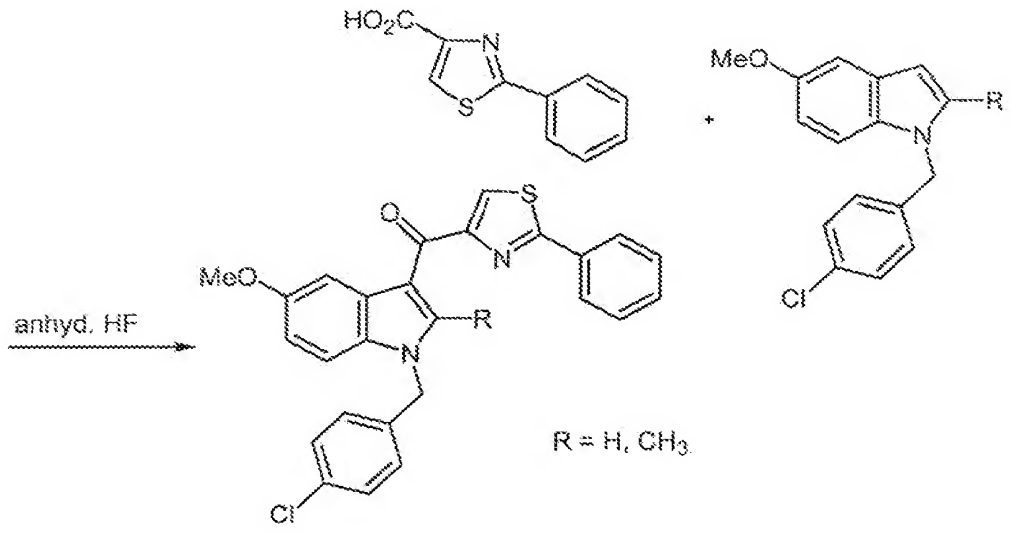
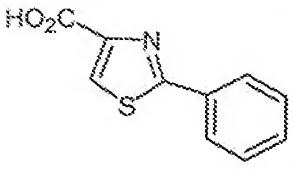
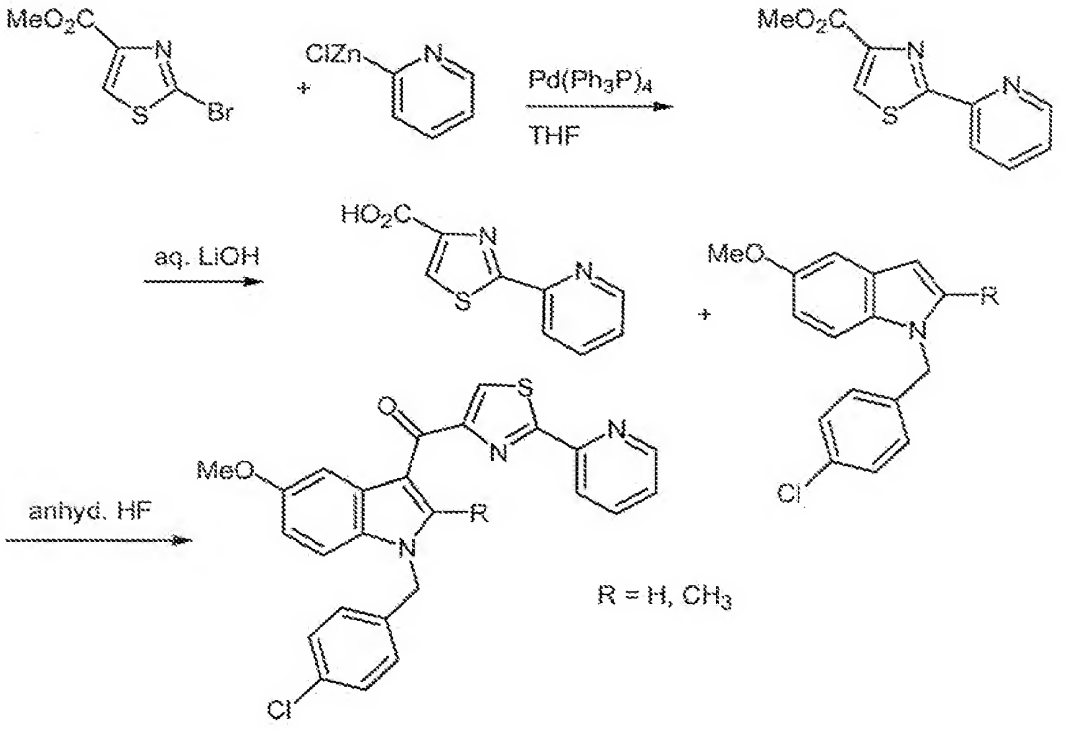
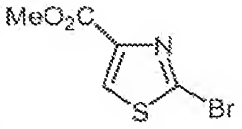


See Hodgetts et al. Org. Lett. 2002, 4, 1363-1365. Starting material

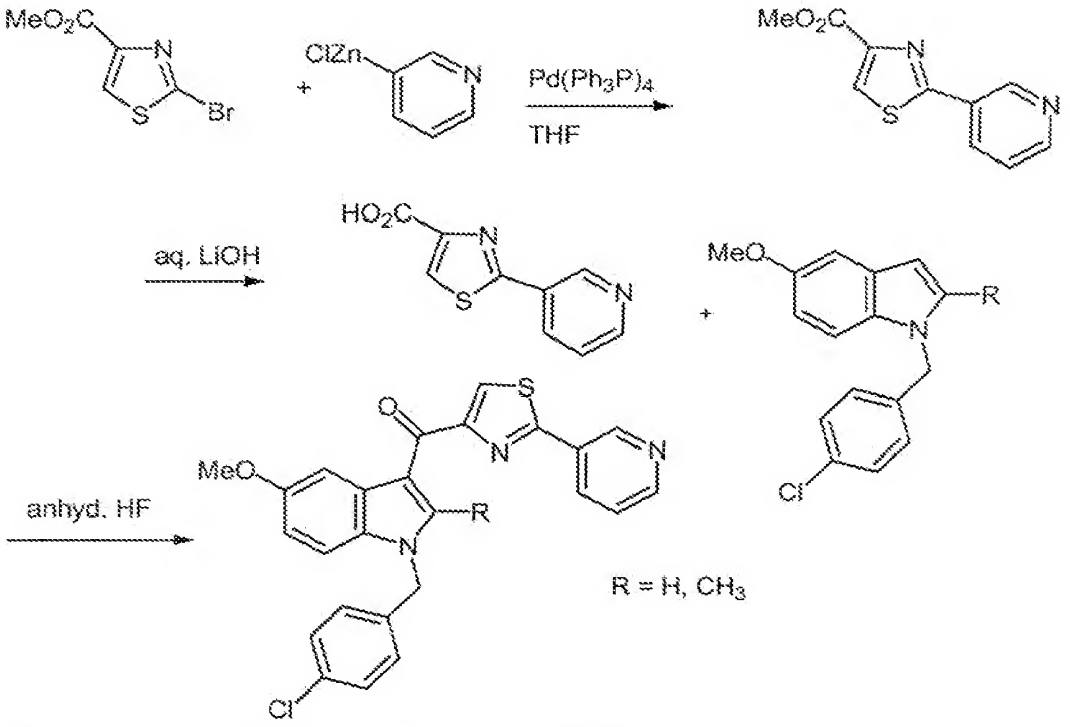


(CA registry no. 7113-10-2) is available from SynChem Inc. (Des Plaines, IL, catalog no. SC-22021)

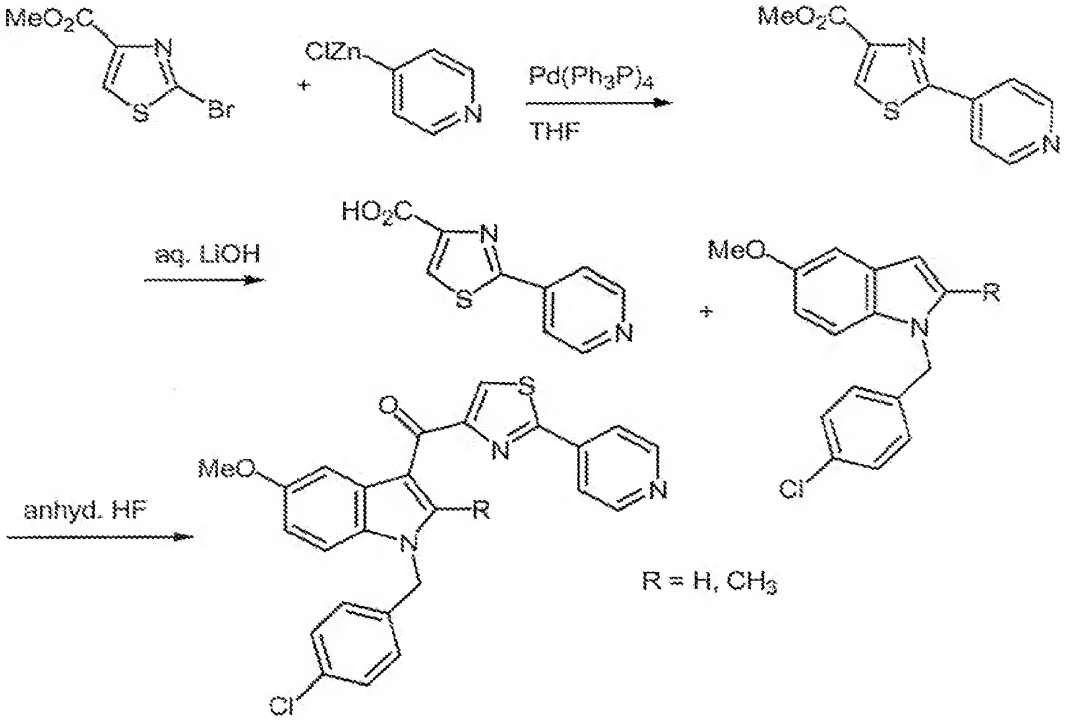




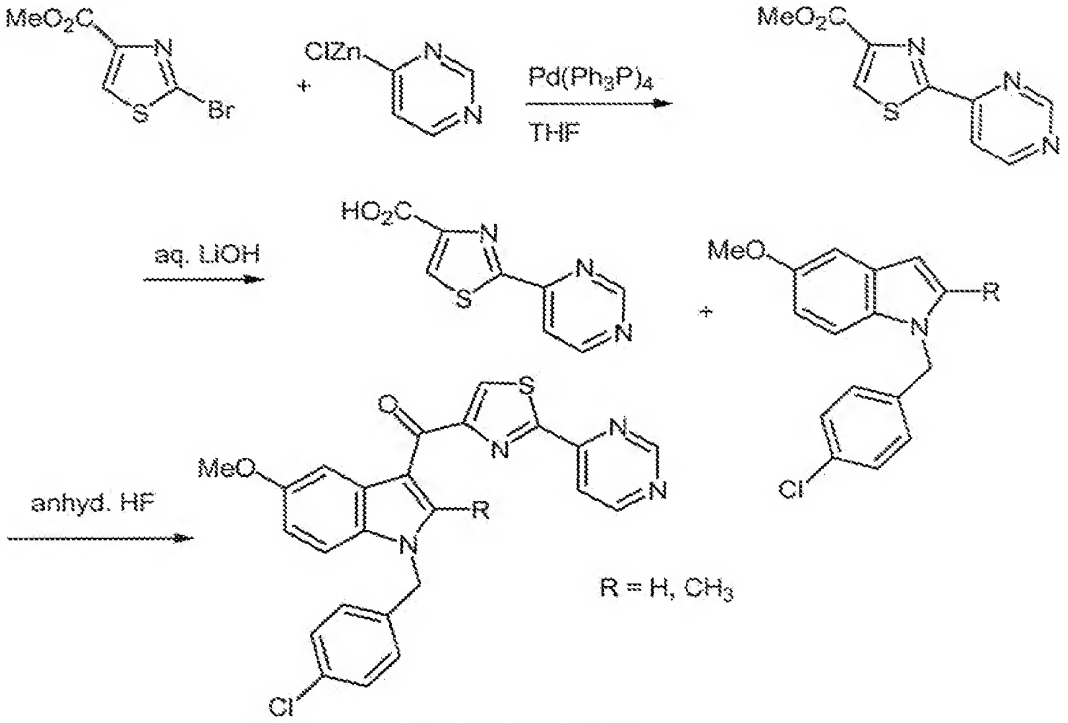
See Hodgetts et al. Org. Lett. 2002, 4, 1363-1365. Starting material (CA registry no. 170235-26-4) is available from SynChem Inc. (Des Plaines, IL, catalog no. SC-21789).



See Hodgetts et al. Org. Lett. 2002, 4, 1363-1365.



See Hodgetts et al. Org. Lett. 2002, 4, 1363-1365.



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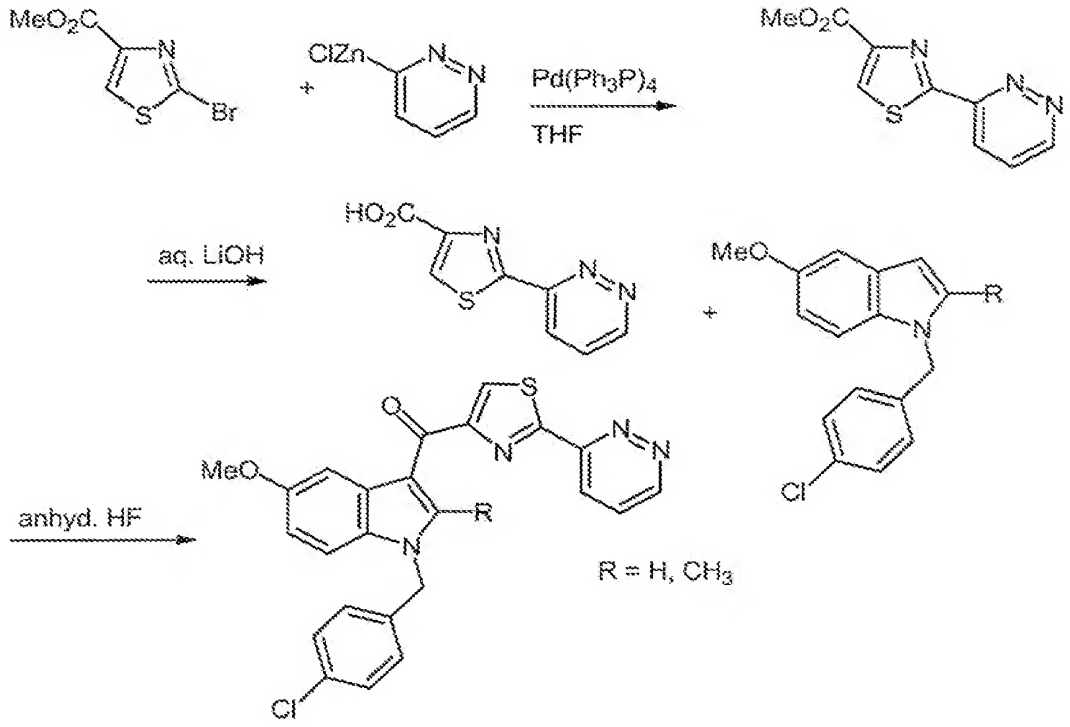
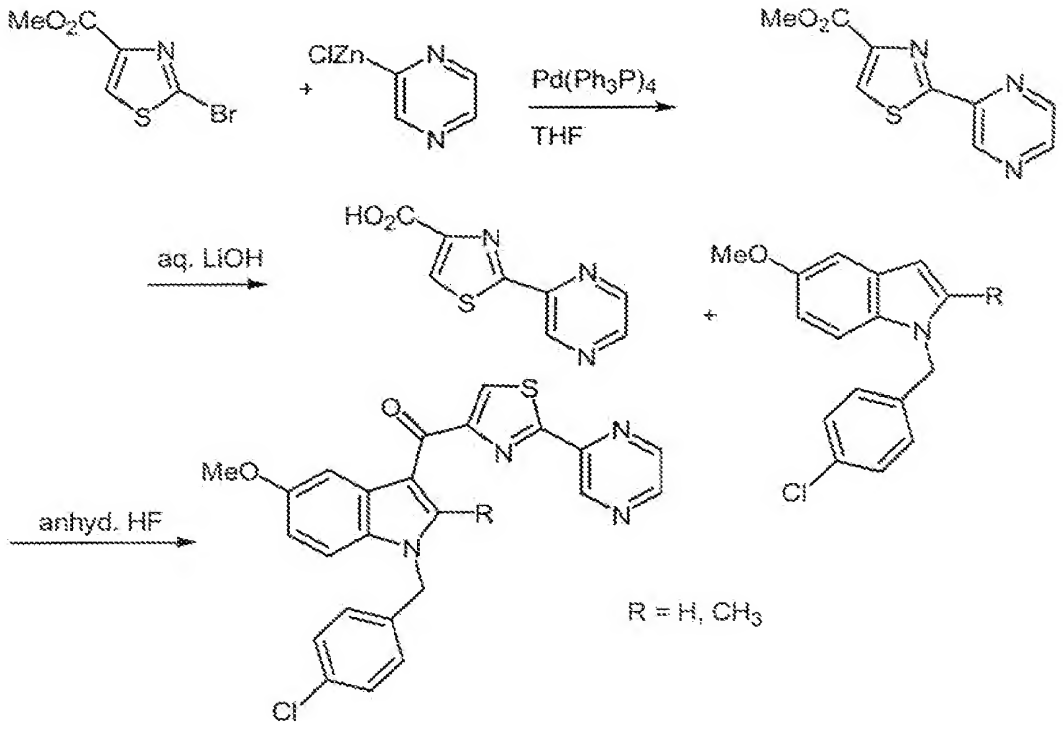
See Hodgetts et al. Org. Lett. 2002, 4, 1363-1365.



See Hodgetts et al. Org. Lett. 2002, 4, 1363-1365.



See Hodgetts et al. Org. Lett. 2002, 4, 1363-1365.

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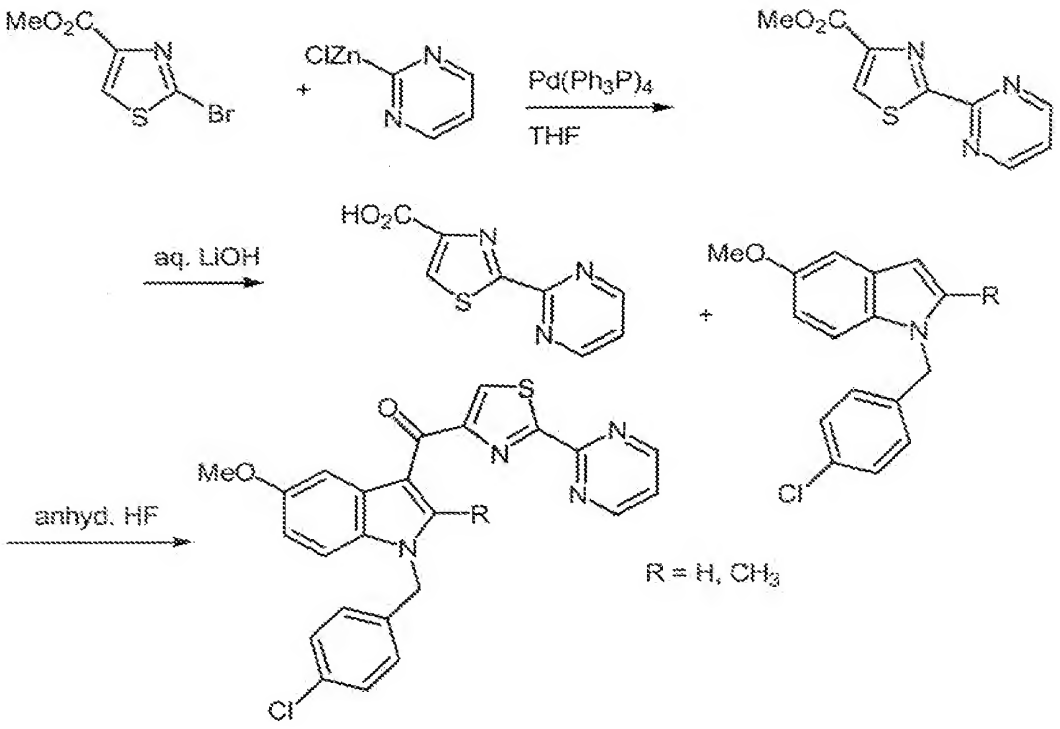

See Hodgetts et al. Org. Lett. 2002, 4, 1363-1365.

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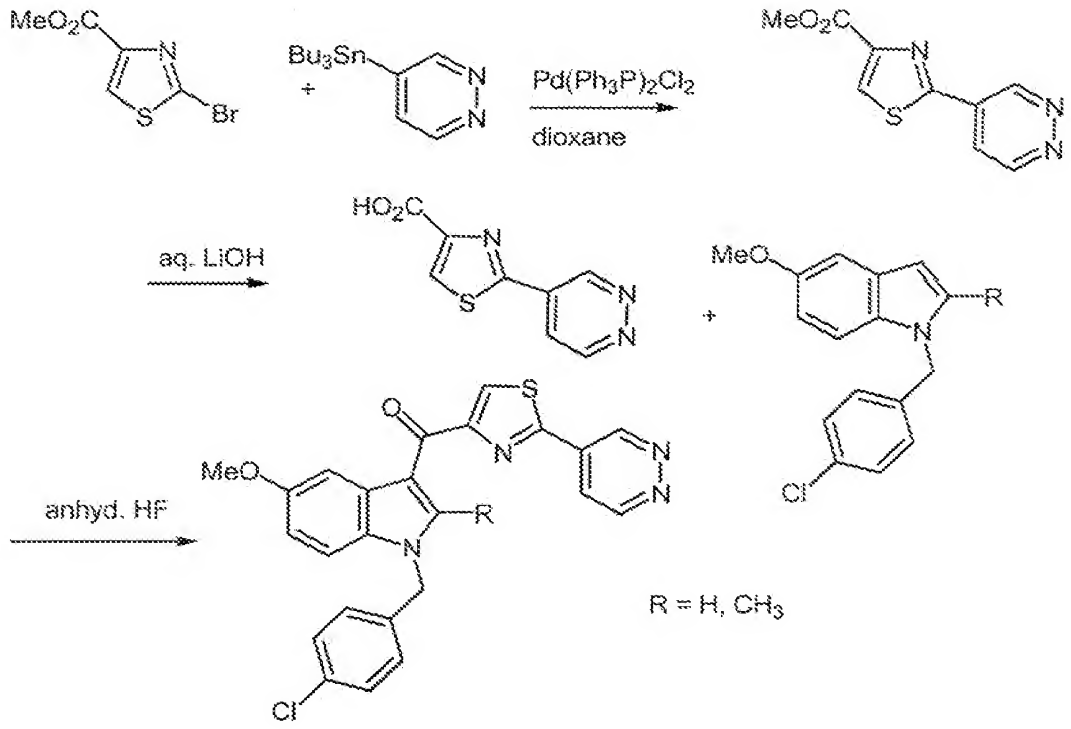
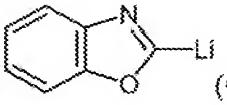
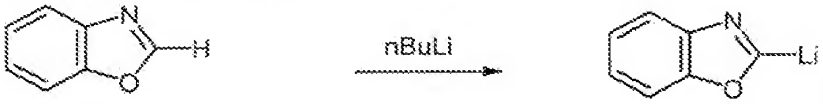


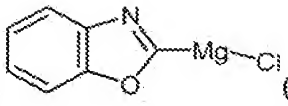
See Hodgetts et al. Org. Lett. 2002, 4, 1363-1365.

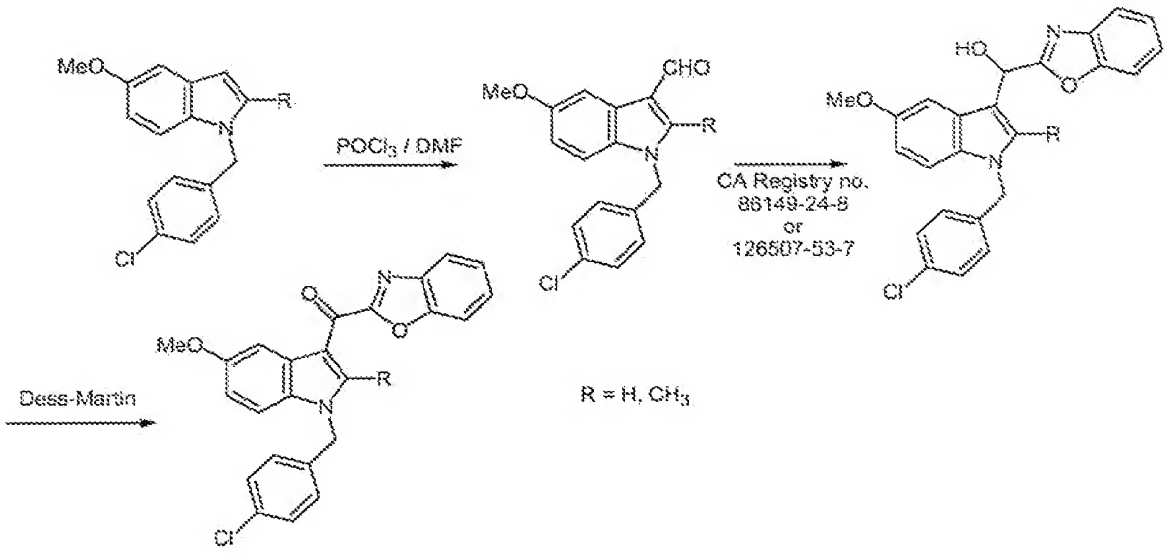
Organo-lithium  (CA Registry no. 86149-24-8) can be synthesized using the following reaction:

CA Registry no. 273-53-0

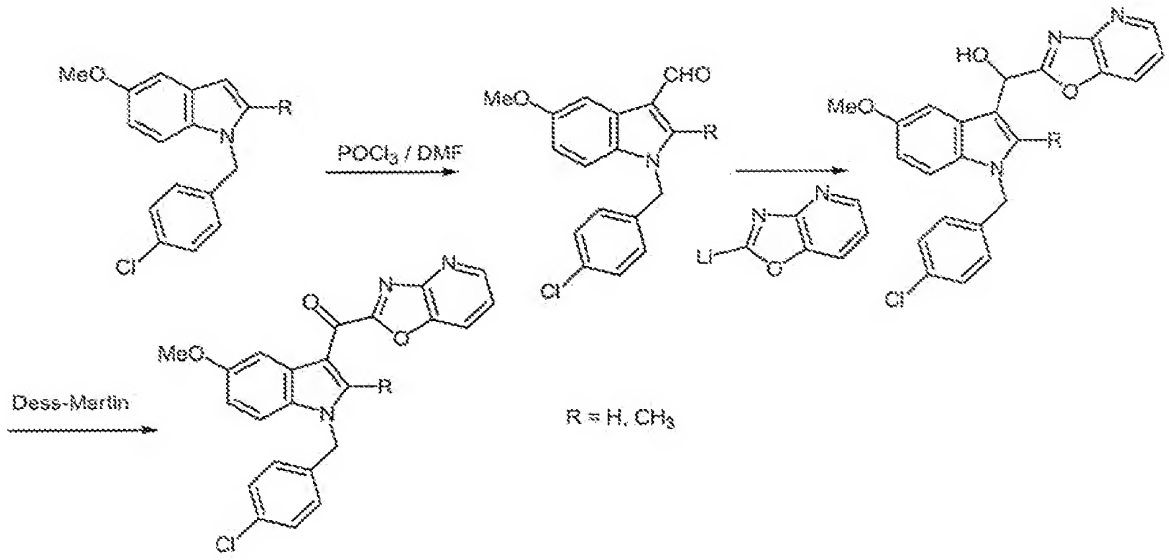


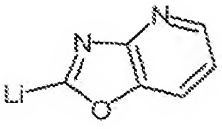
as described in Subramanyam and Chang Tetrahedron Letters 2002, 43, 6313-6315. CA Registry 273-53-0 is commercially available (Alfa Aesar, Ward Hill, MA, catalog no. A17489)

Grignard reagent  (CA Registry no. 126507-53-7) can be prepared using the methods described in WO2003087068. Although in most cases the organo-lithium derivative is shown, both organo-lithium and Grignard reagents can be prepared and used similarly in the subsequent 5 schema.



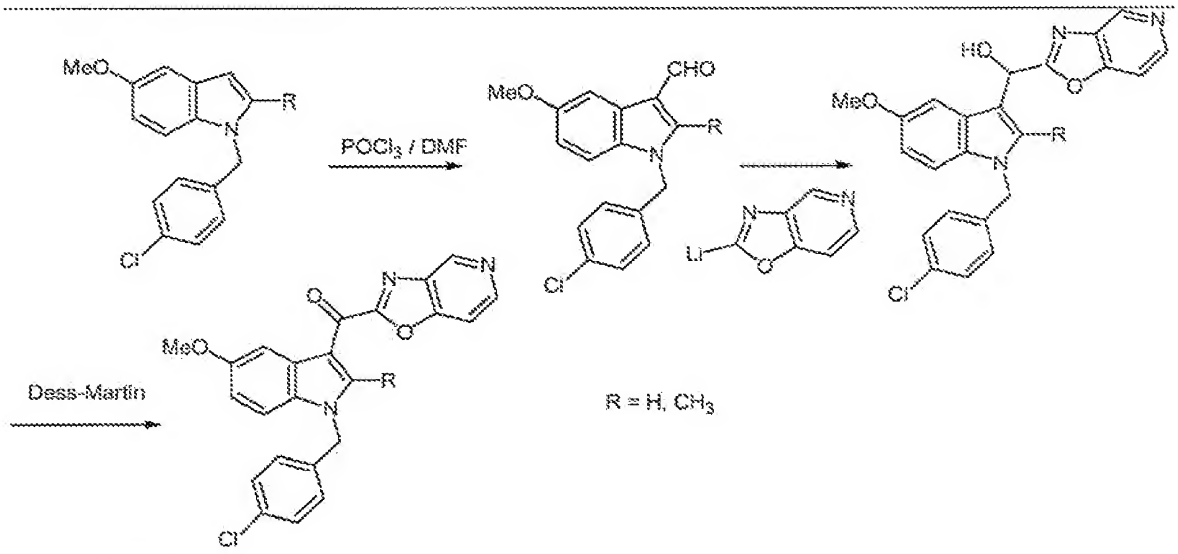
See Boger et al. J. Med. Chem. 2005, 48, 1849-1856.





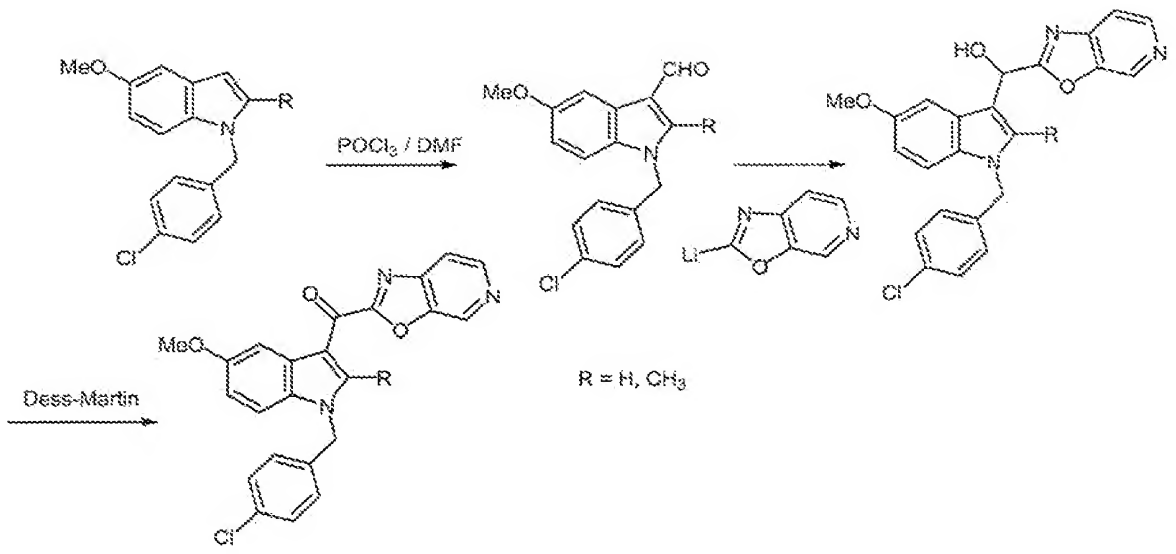
is prepared as described in WO2003087068.

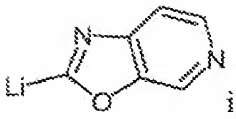
See Boger et al. J. Med. Chem. 2005, 48, 1849-1856 for general reaction schema.



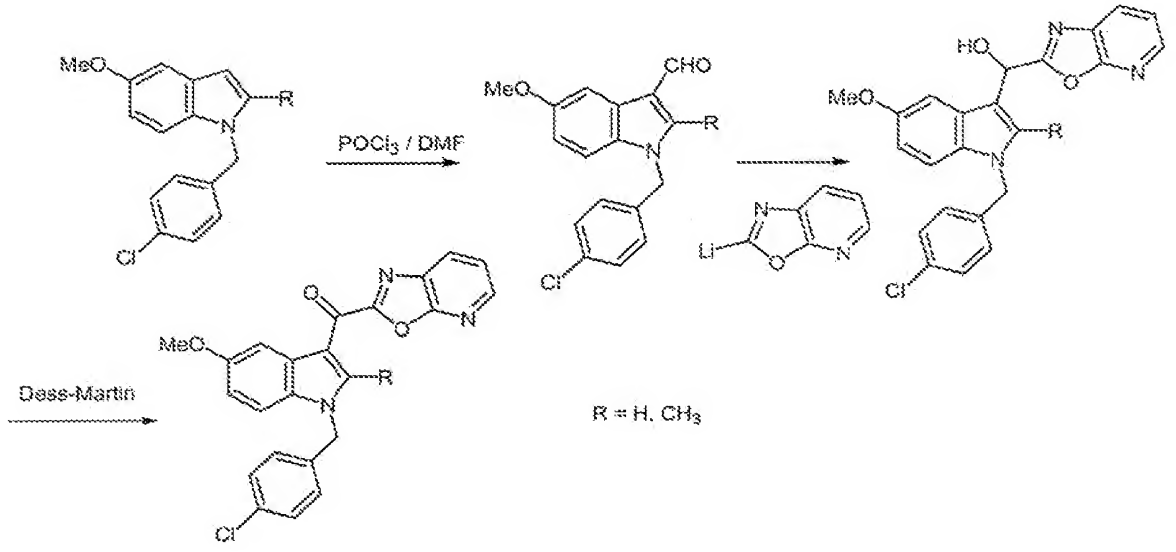
(CA Registry no. 273-56-3) can be synthesized using the processes described in Katner and Brown Journal of Heterocyclic Chemistry 1990, 27, 563-536 and an organo-lithium derivative or Grignard derivative prepared as described above.

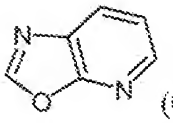
See Boger et al. J. Med. Chem. 2005, 48, 1849-1856 for general reaction schema.

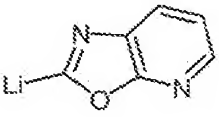



is prepared as described in Heuser et al. Tetrahedron Letters 2005, 46, 9001-9004.

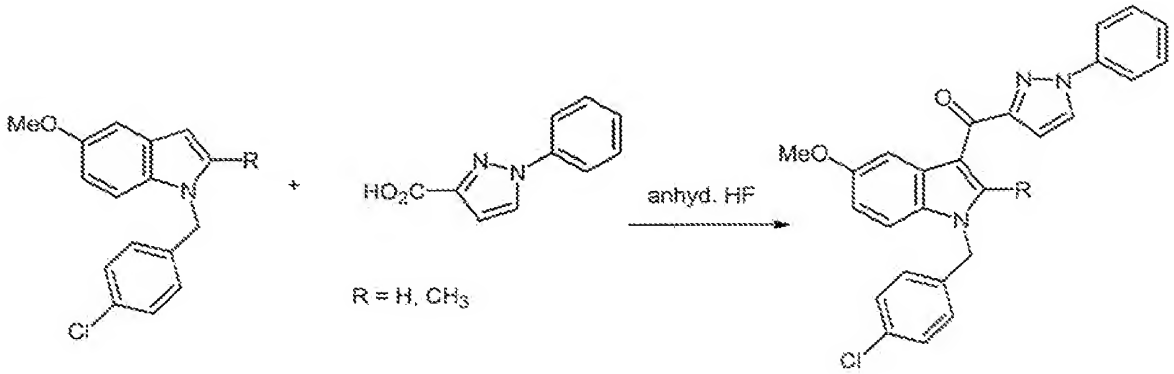
See Boger et al. J. Med. Chem. 2005, 48, 1849-1856 for general reaction schema.

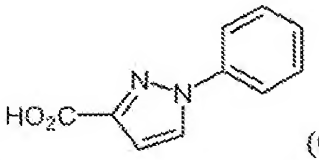


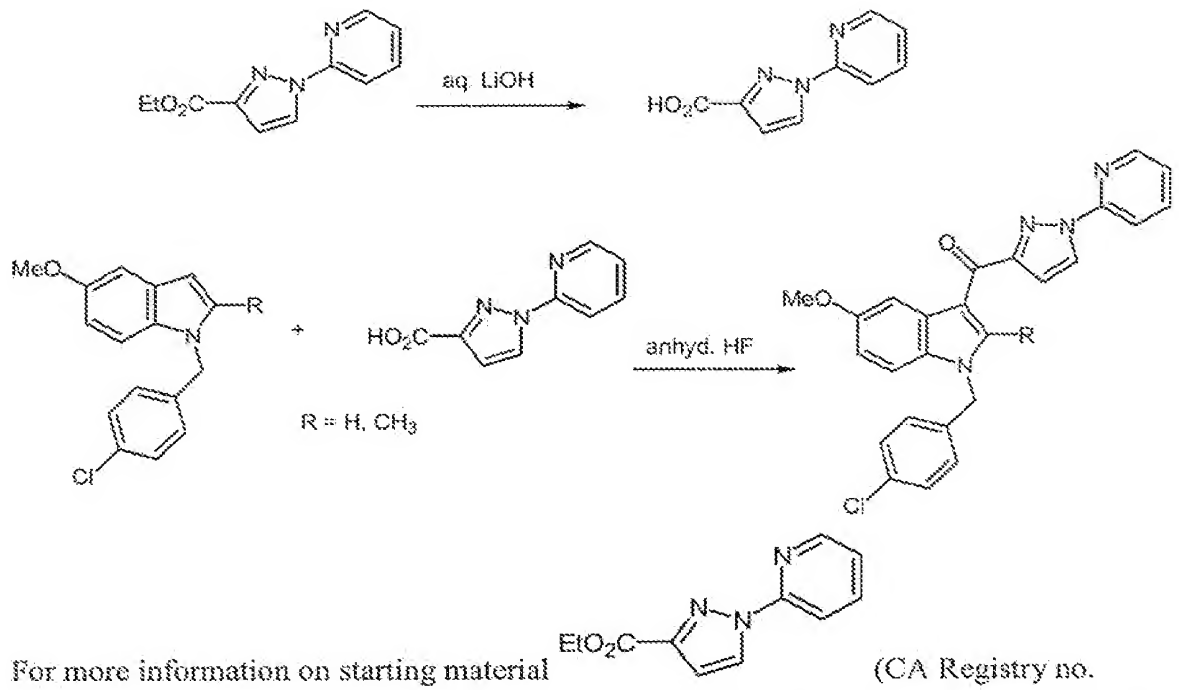

(CA Registry no. 273-62-1) is prepared as described in Takahashi and Koshiro, Chemical & Pharmaceutical Bulletin 1959, 7, 720-725 and the organo-lithium derivative,


is prepared as described above.

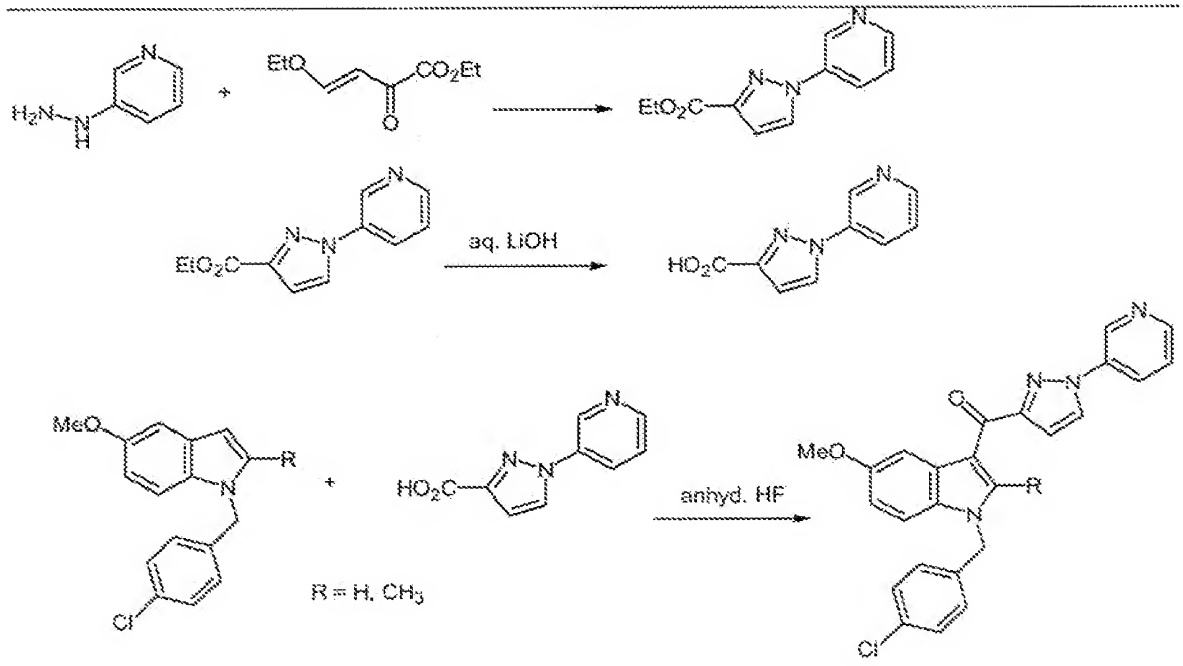
See Boger et al. J. Med. Chem. 2005, 48, 1849-1856 for general reaction schema.

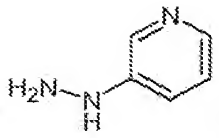


For more information on starting material  (CA registry no. 4747-46-0) see Tabak et al. Tetrahedron 1966, 22, 2703-2710.



For more information on starting material (CA Registry no. 154012-24-5) see Holzer and Seiringer Journal of Heterocyclic Chemistry 1993, 30, 865-872.



Starting material  (CA Registry No. 42166-50-7) is available from Beta Pharma, Inc. (New Haven, CT, catalog no. 23097)

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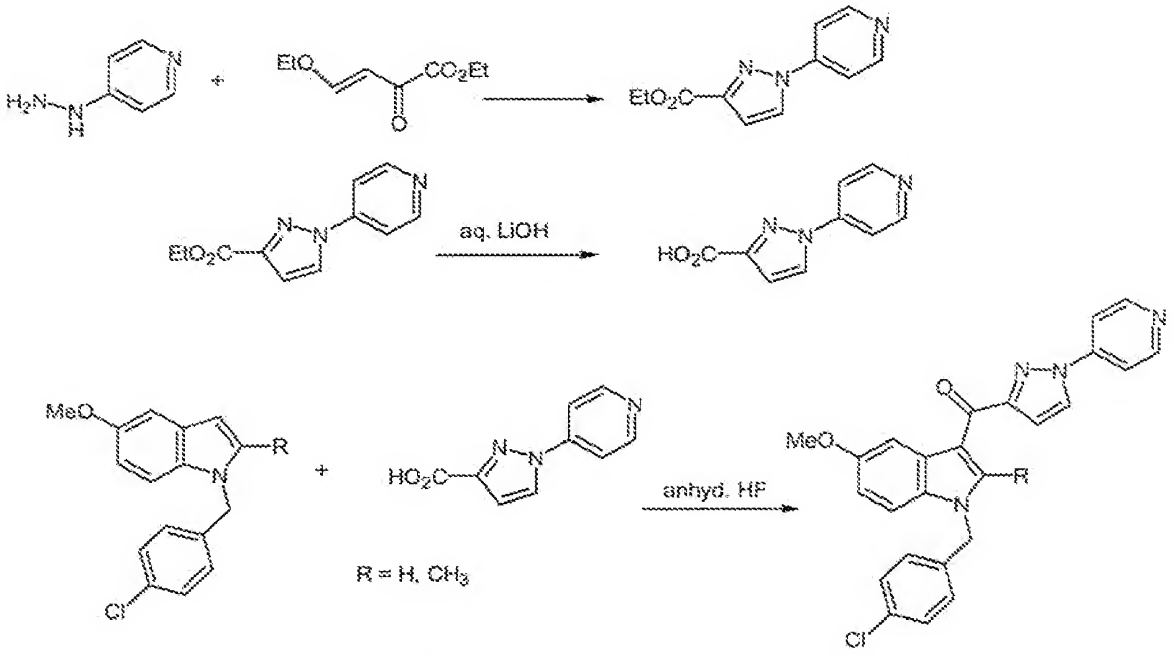
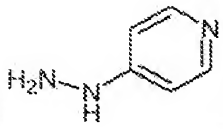
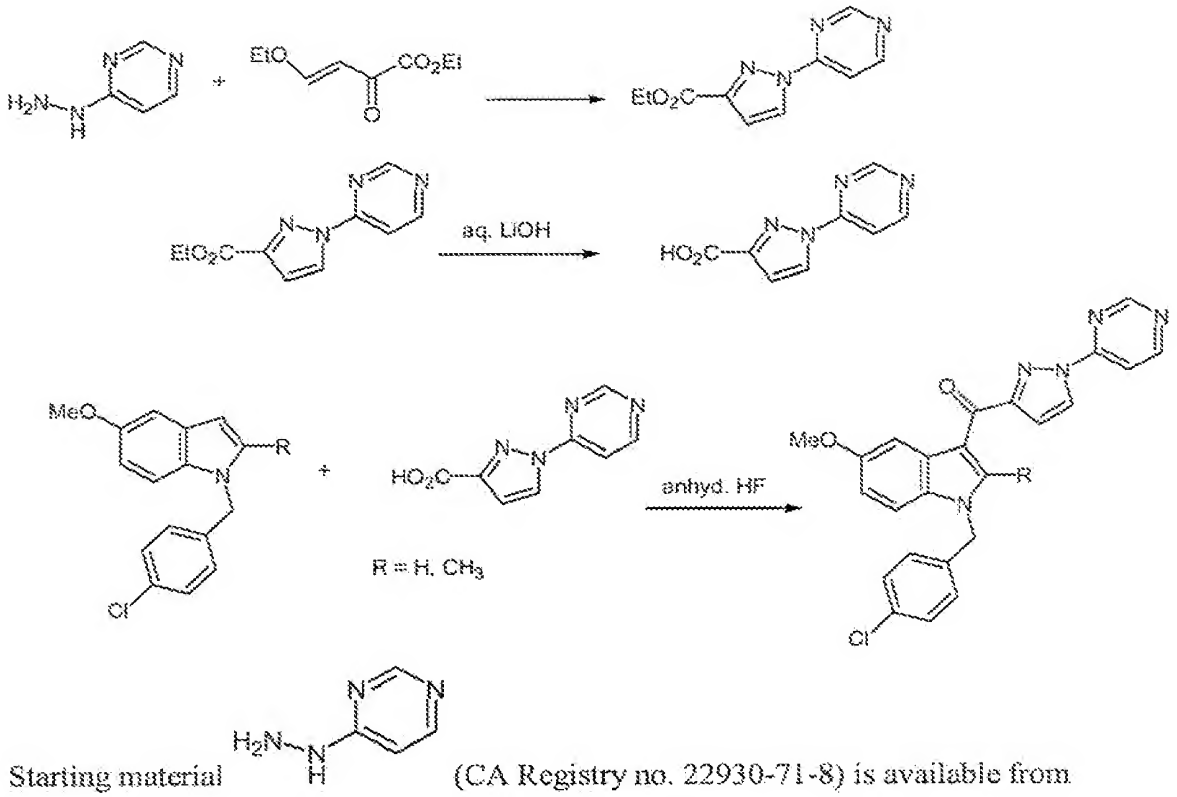
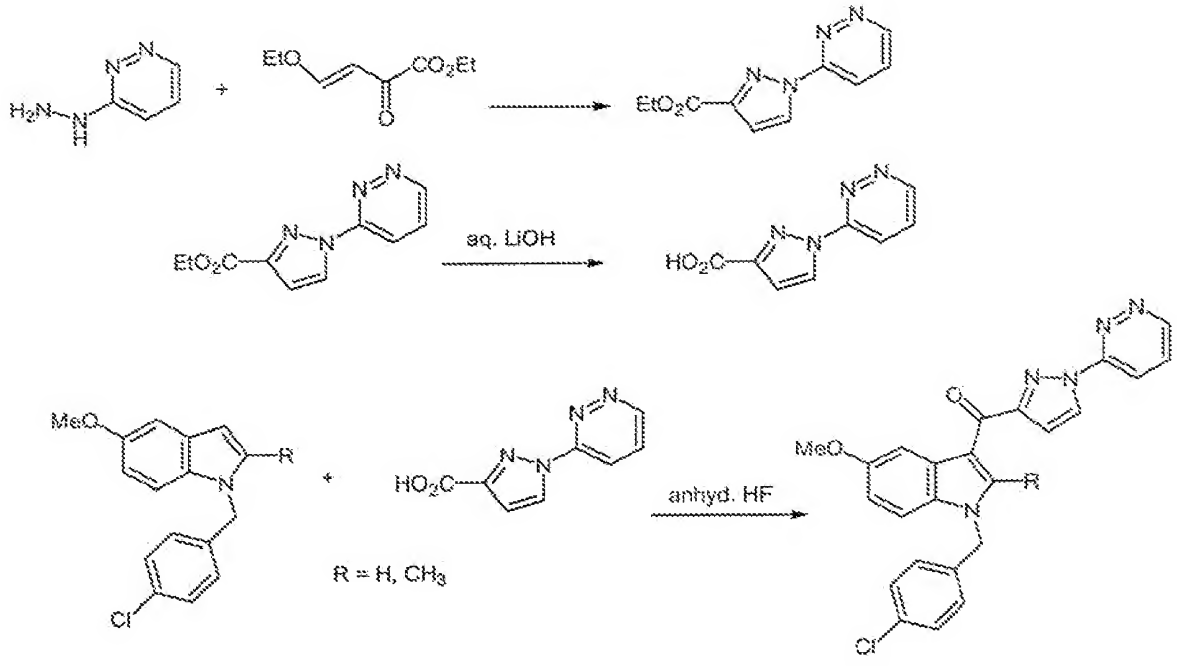
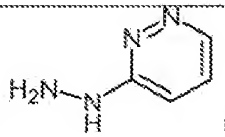


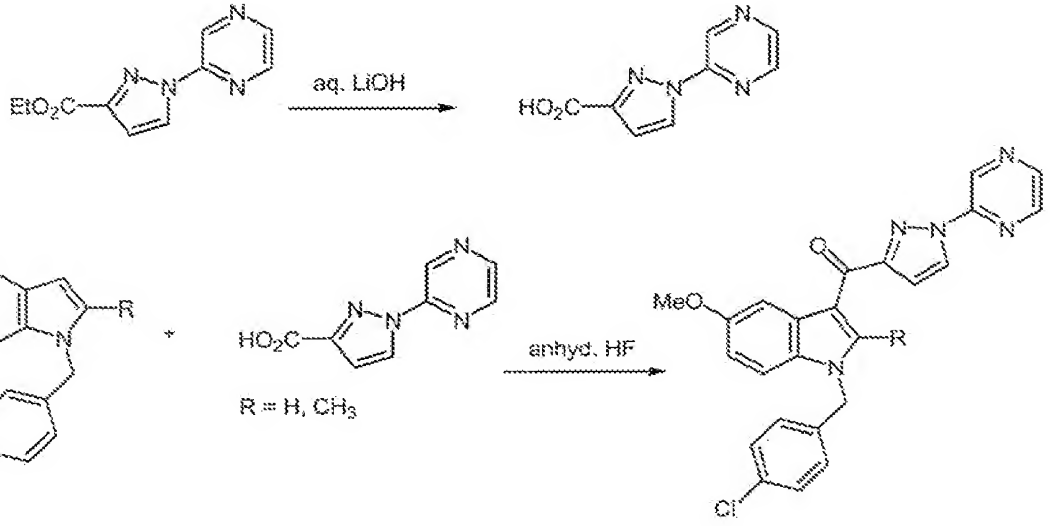
Starting material  (CA Registry no. 20815-52-5) is available from Prime Organics, Inc. (Lowell, MA, catalog no. POI-58-21)

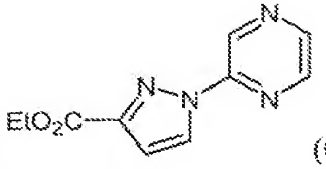


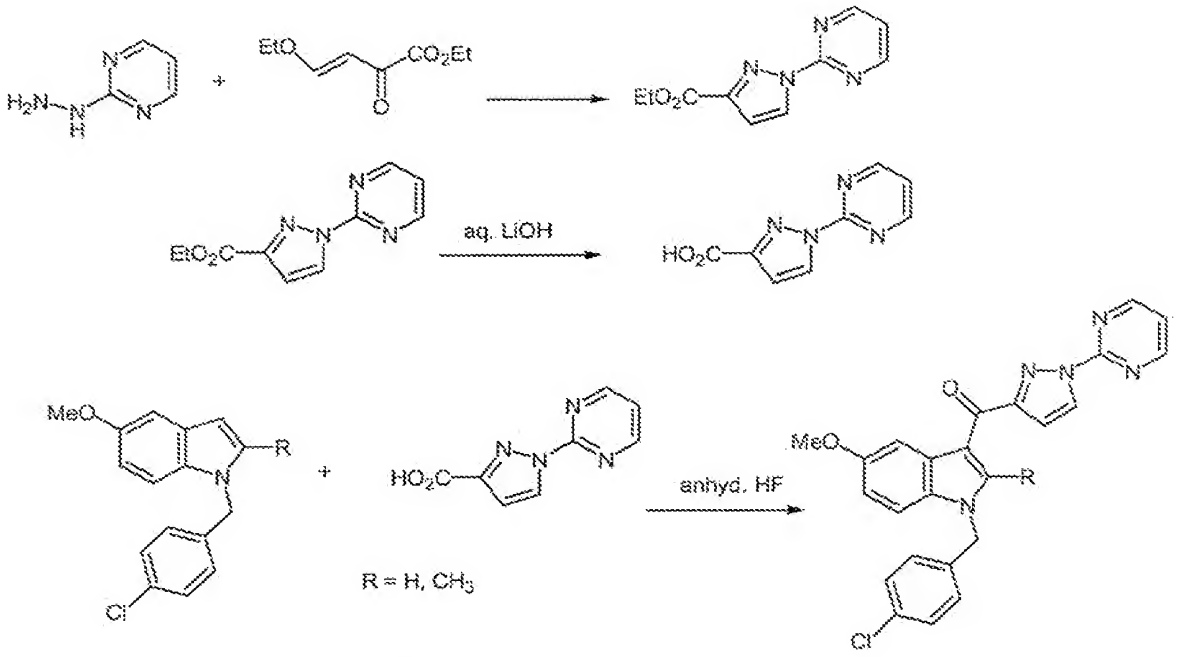
Starting material (CA Registry no. 22930-71-8) is available from Chemstep (Carbon Blanc, France, catalog no. 19541)

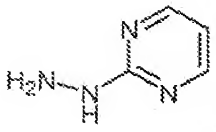


For more information on starting material  (CA Registry no. 40972-16-5) see Pinza and Pifferi Farmaco 1994, 49, 683-692.



For more information on starting material  (CA Registry no. 154012-27-8) see Holzer and Seiringer Journal of Heterocyclic Chemistry 1993, 30, 865-872.



Starting material  (CA Registry no. 7504-94-1) is available from Scientific Exchange, Inc. (Center Ossipee, NH, catalog no. M-278979)

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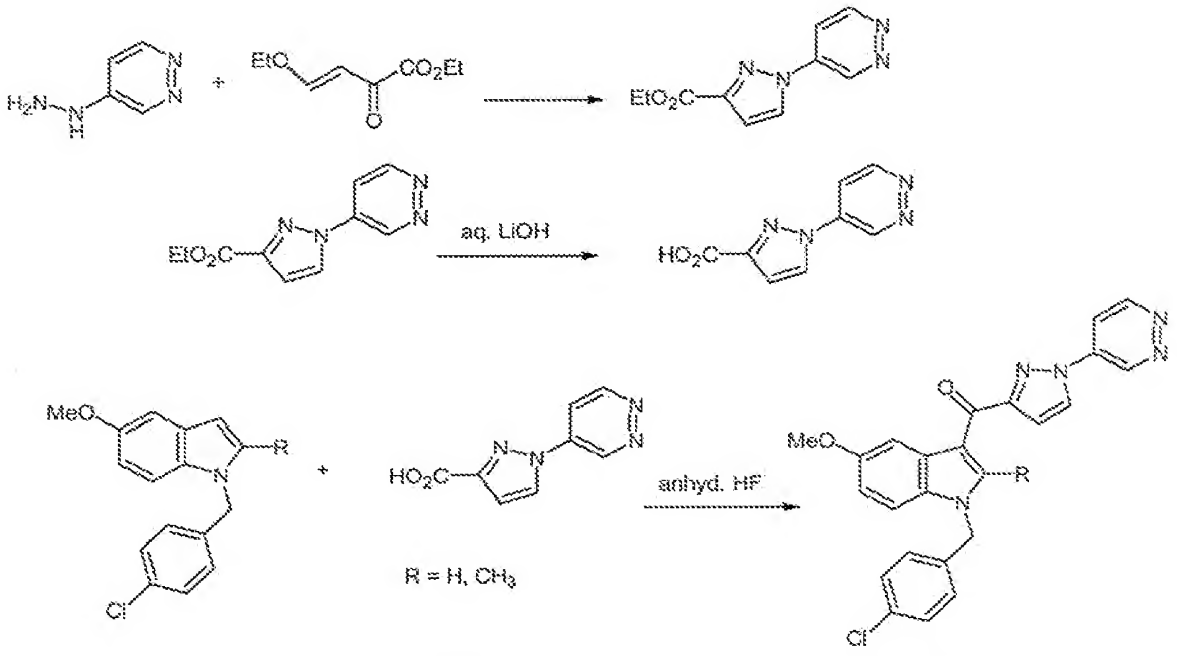
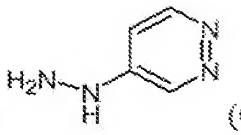
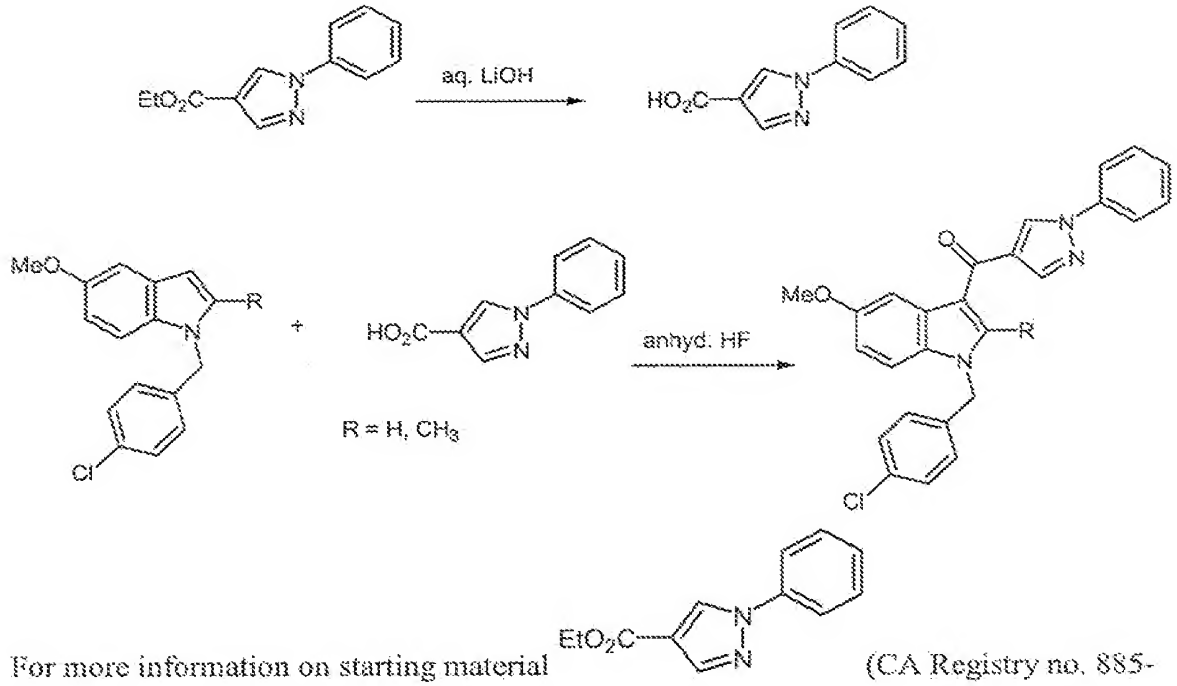
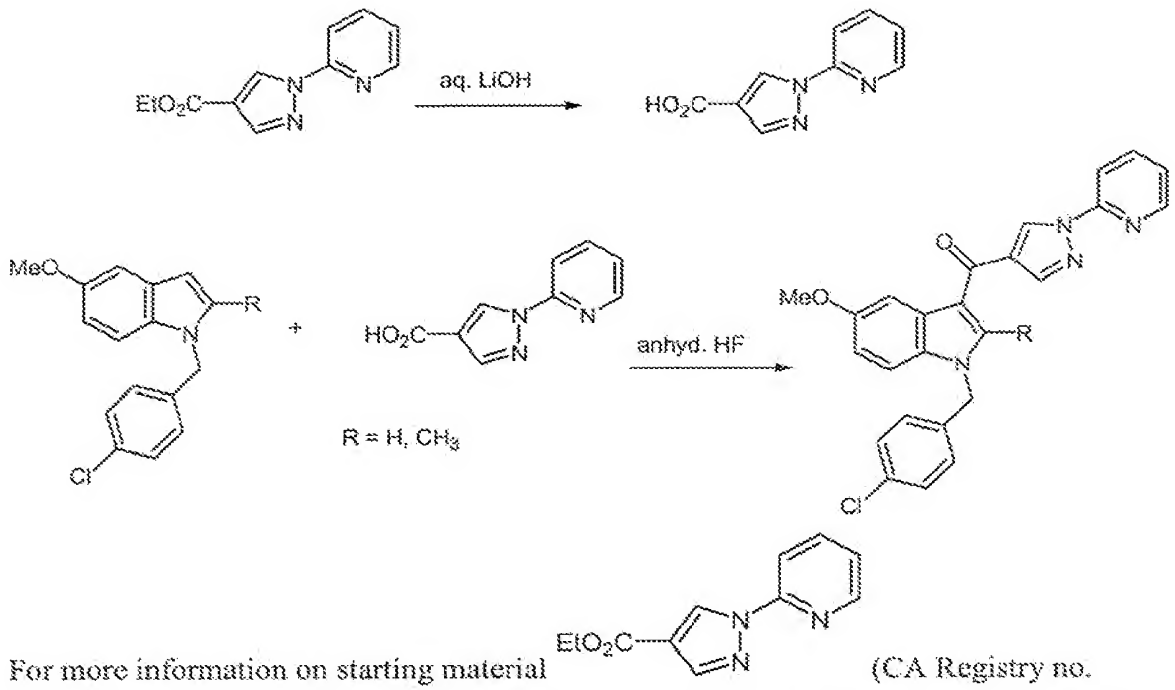


For more information on starting material  (CA Registry No. 103394-79-2) see Schuler and Wyss, E. Archives Internationales de Pharmacodynamie et de Therapie 1960, 128, 431-468.



For more information on starting material (CA Registry no. 885-94-9) see Holzer and Seiringer Journal of Heterocyclic Chemistry 1993, 30, 865-872.



For more information on starting material (CA Registry no. 171193-35-4) see See: Holzer and Schmid Journal of Heterocyclic Chemistry 1995, 32, 1341-1349.

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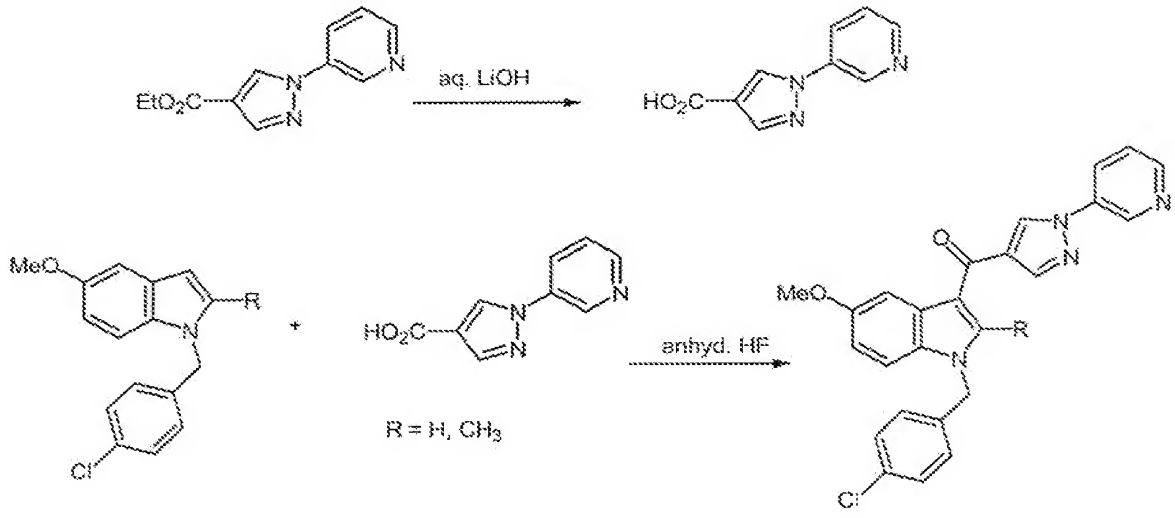
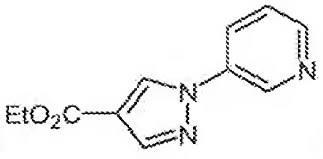
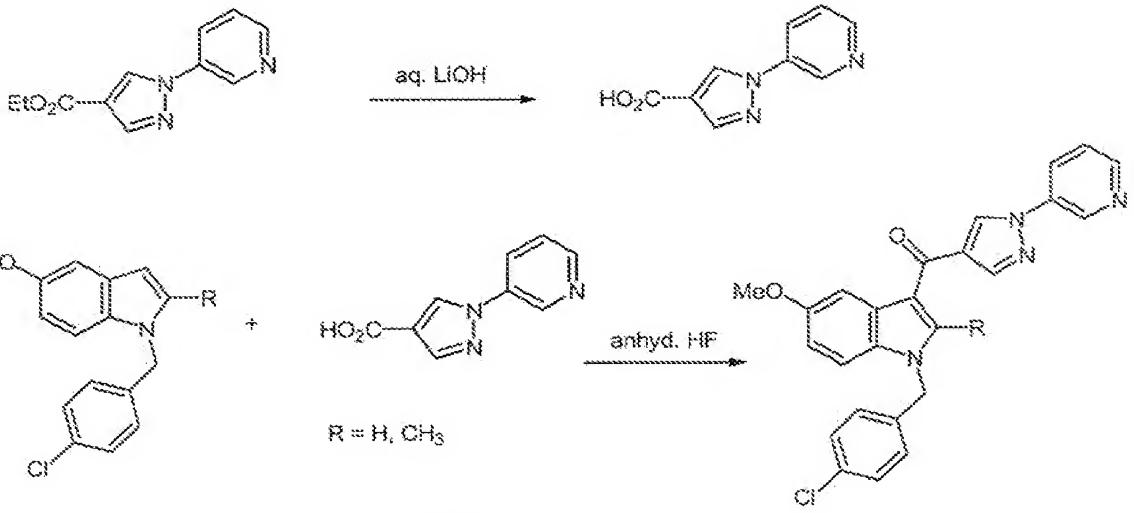
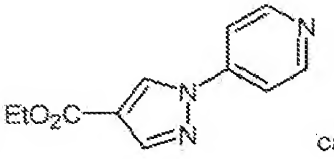
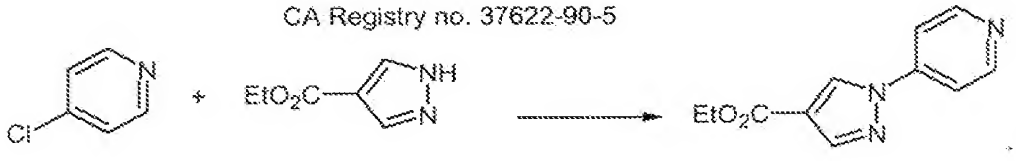


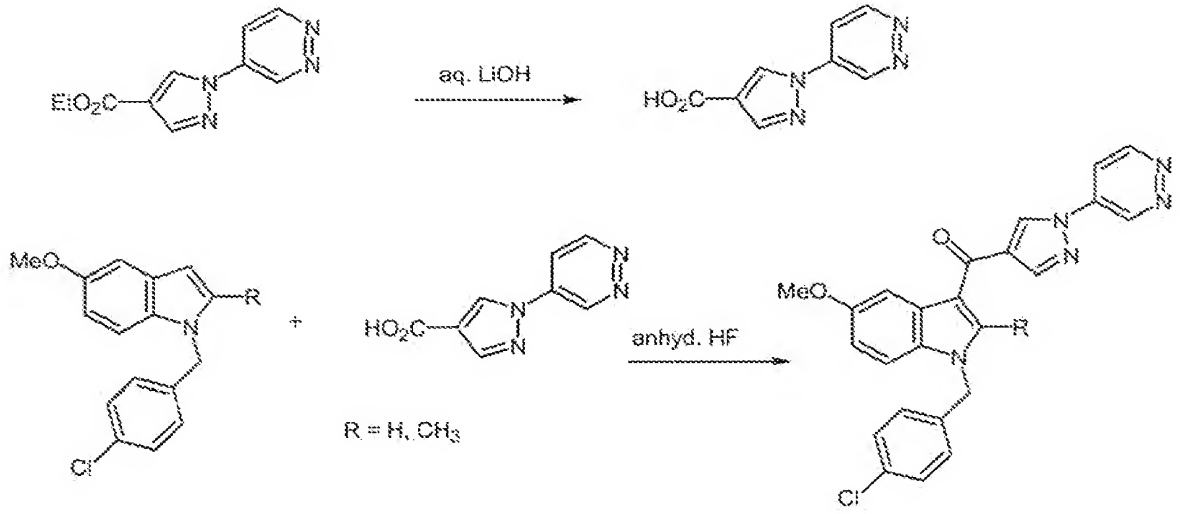
For more information on starting material  (CA Registry No. 741717-60-2) see Antilla et al. Journal of Organic Chemistry 2004, 69, 5578-5587.

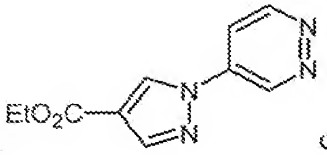


Starting material  can be synthesized from the following reaction:

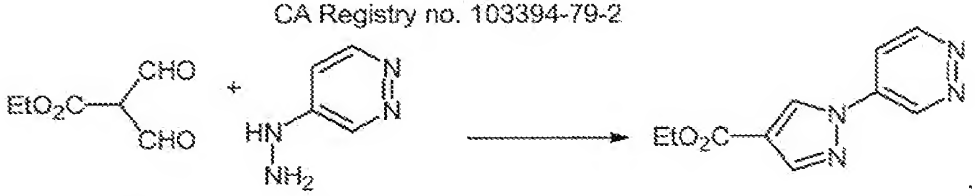


. See Antilla et al. Journal of Organic Chemistry 2004, 69, 5578-5587 for more information.

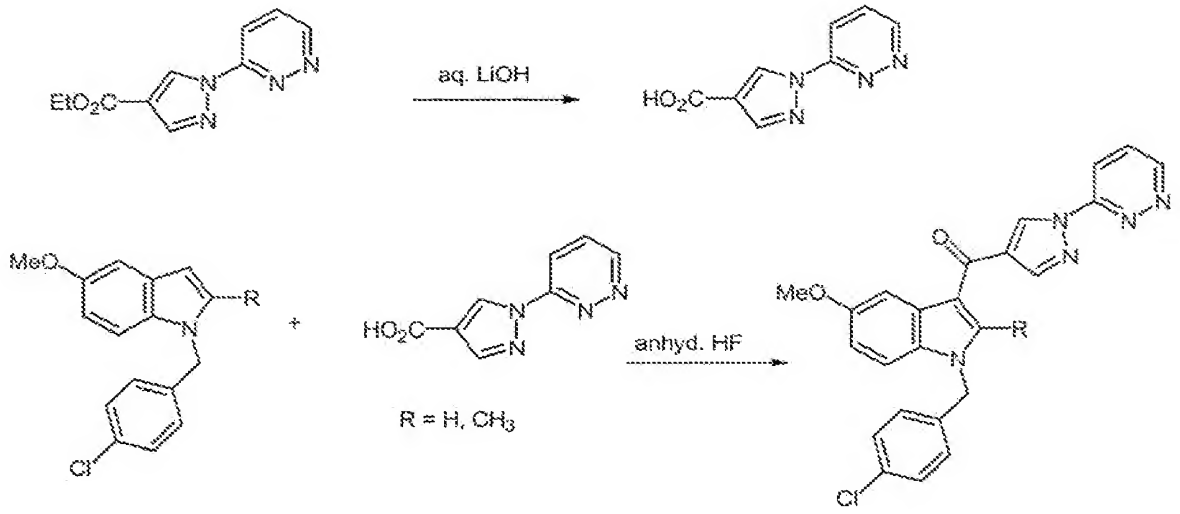


Starting material  can be synthesized from the following reaction:

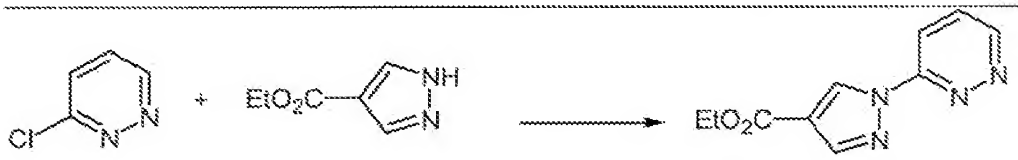
CA Registry no. 103394-79-2



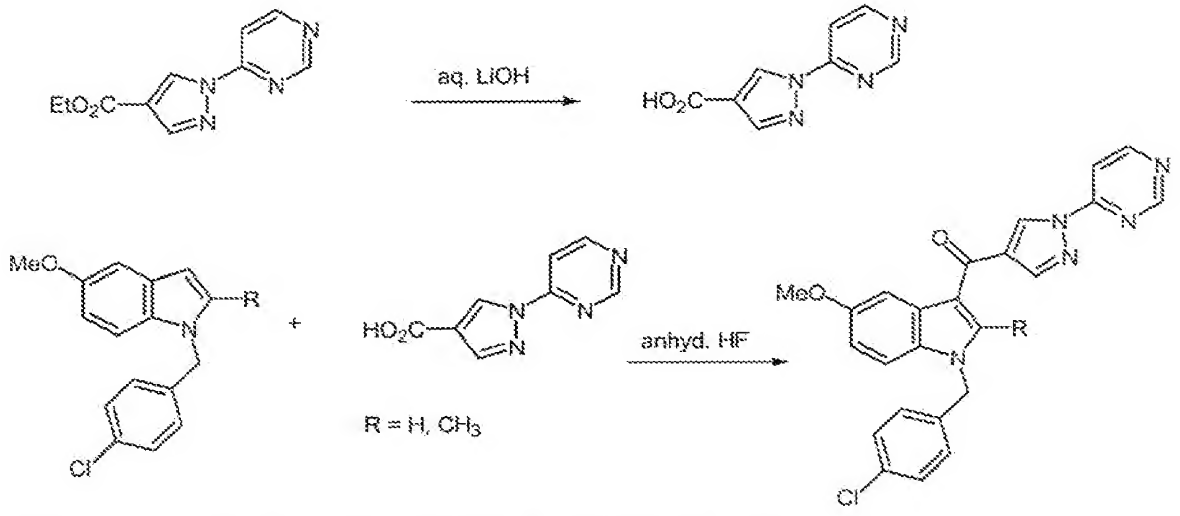
. See Schuler et al. Archives Internationales e Pharmacodynamie et de Therapie 1960, 128, 431-468 for more information.



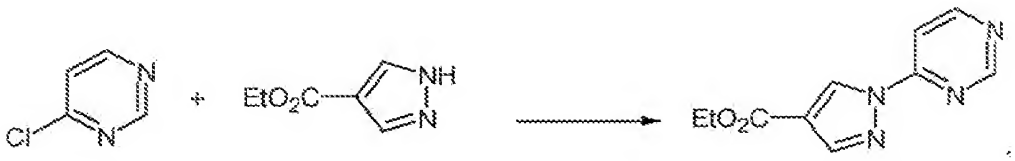
Starting material can be synthesized in the following manner:



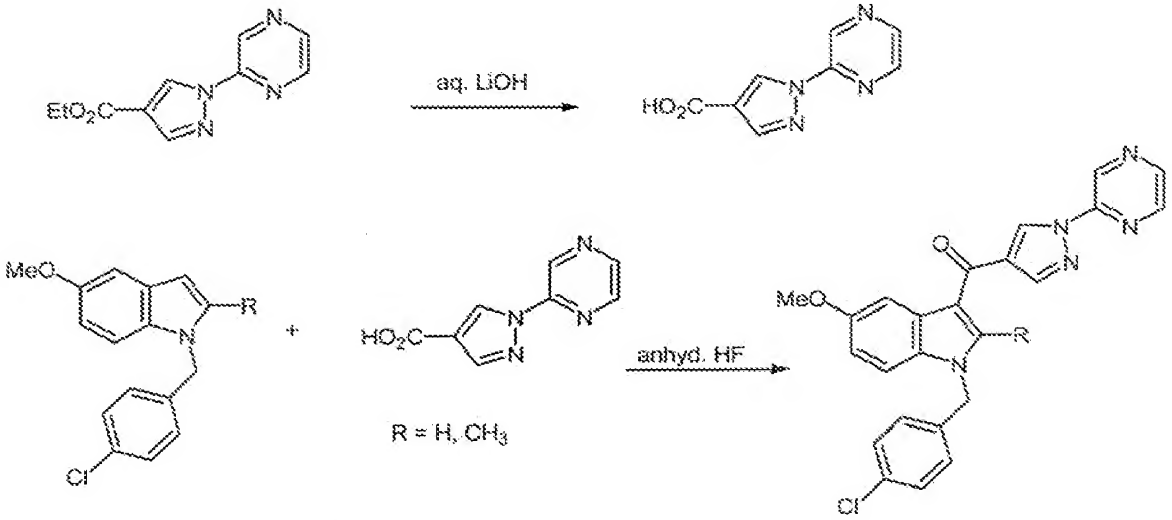
See Antilla et al. Journal of Organic Chemistry 2004, 69, 5578-5587 for more information.



Starting material can be synthesized in the following manner:



See Antilla et al. Journal of Organic Chemistry 2004, 69, 5578-5587 for more information.



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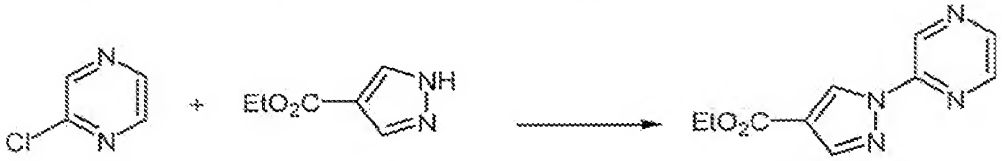
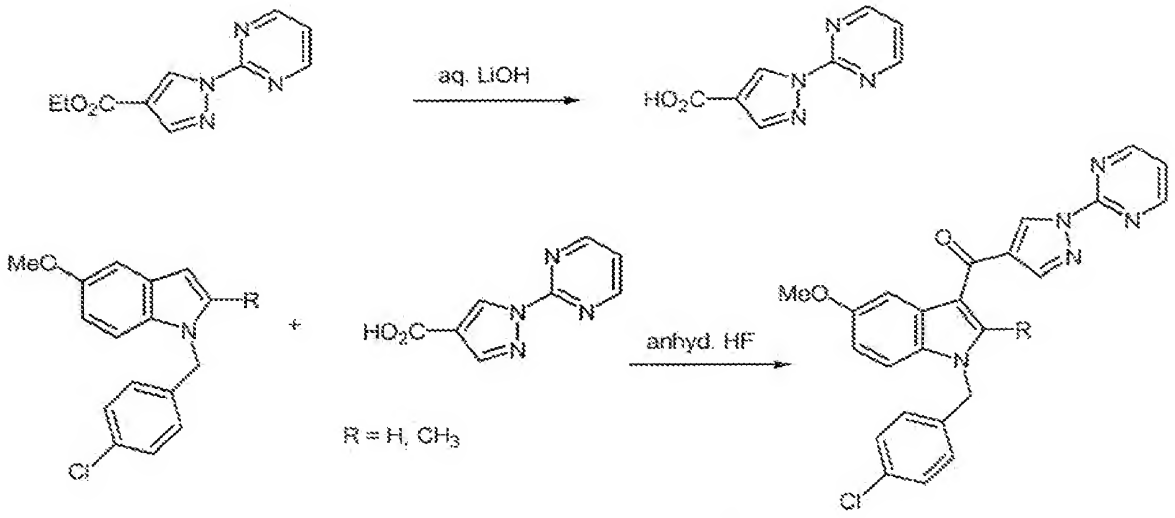


See: Antilla et al. Journal of Organic Chemistry 2004, 69, 5578-5587 for more information.



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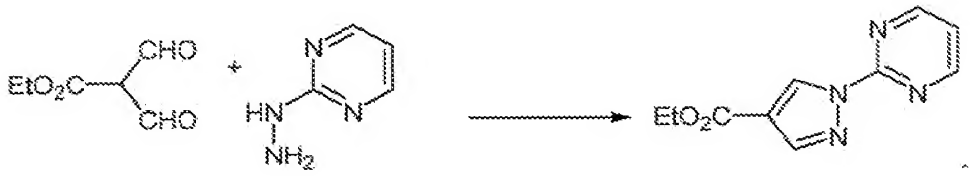

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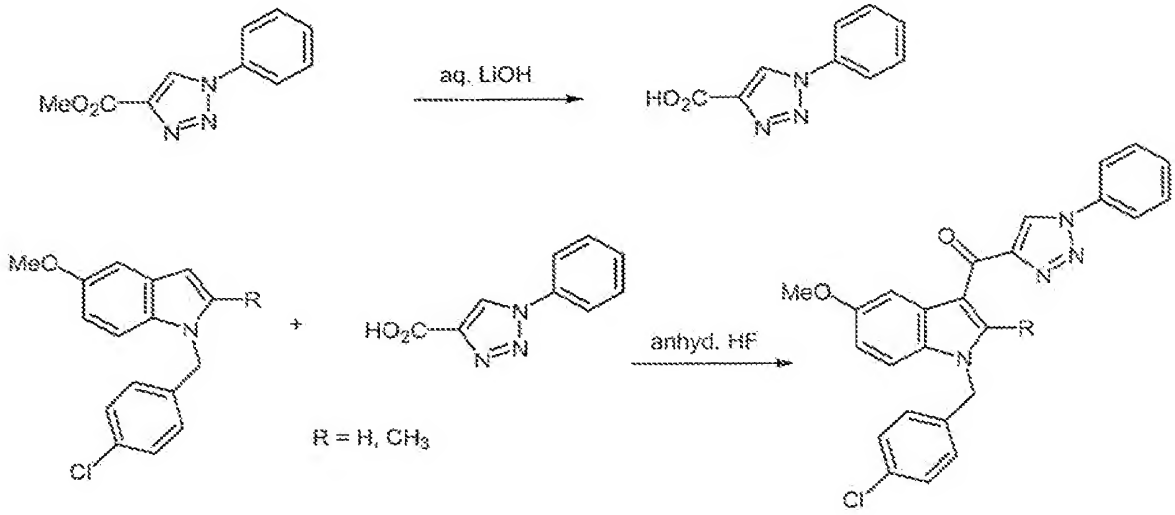
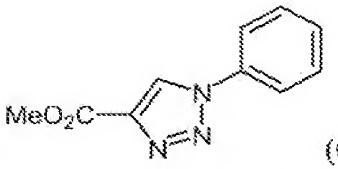
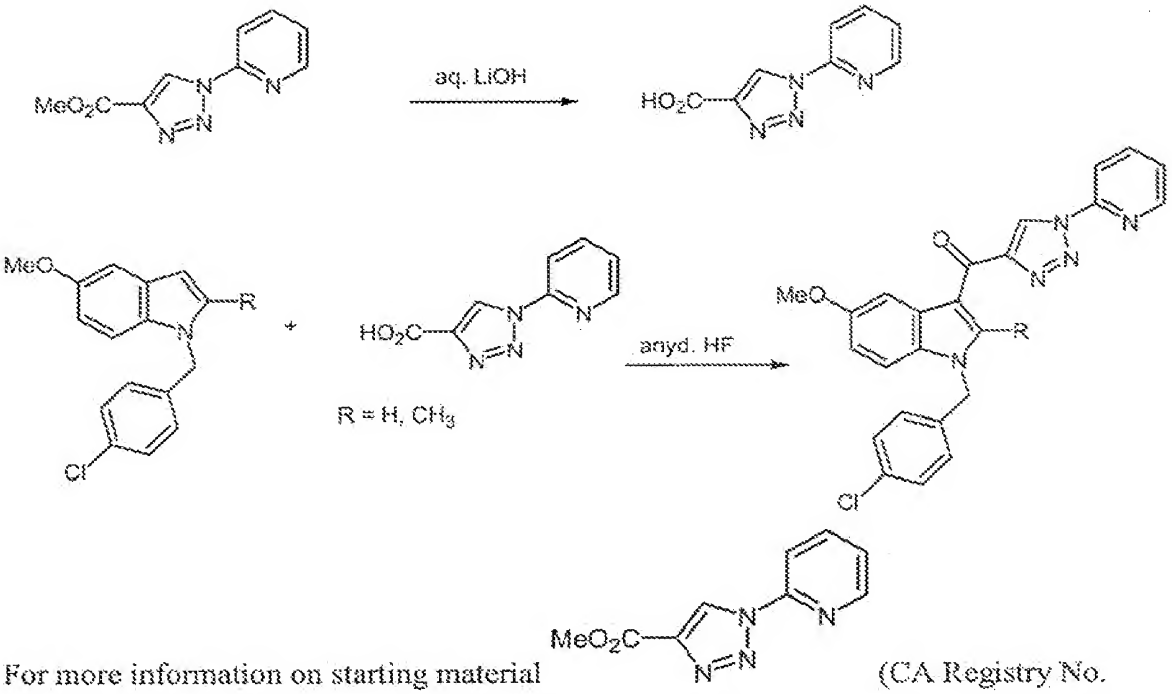
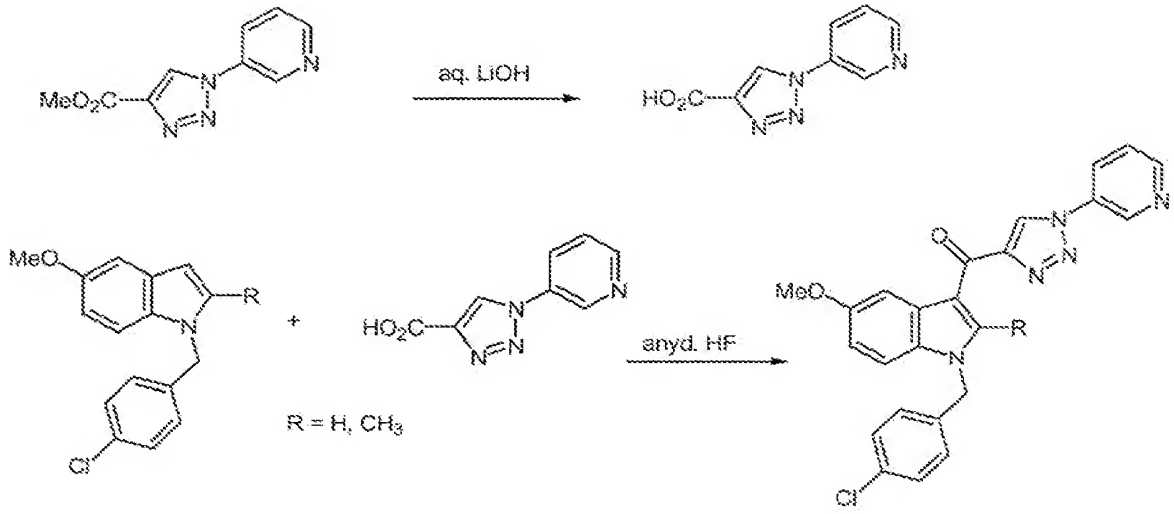




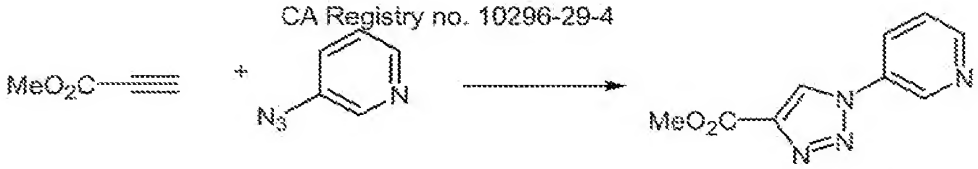
for more information on starting material (CA Registry no. 2055-52-9) see Huisgen et al. Chemische Berichte 1965, 98, 4014-4021 and Fulloon et al. Journal of Organic Chemistry 1996, 61, 1363-1368.



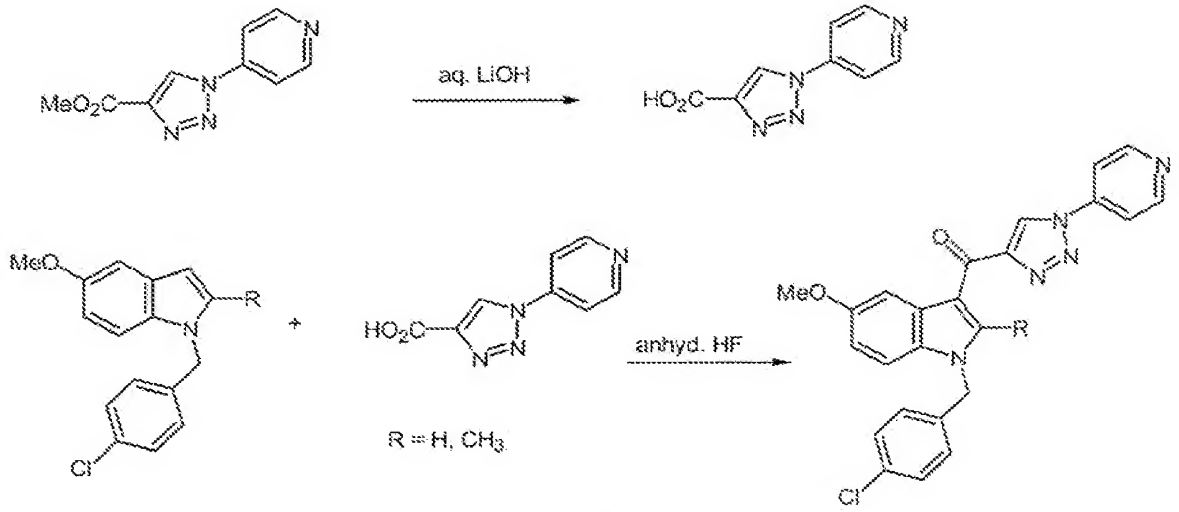
For more information on starting material (CA Registry No. 23947-14-0) see Huisgen et al. Tetrahedron Letters 1969, 30, 2589-2594.



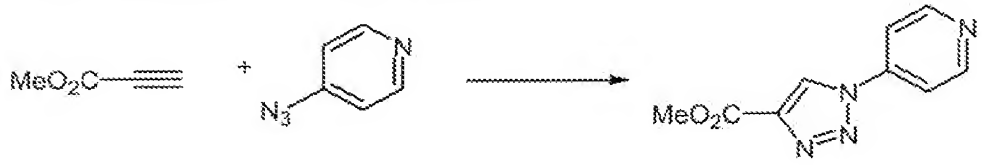
Starting material can be synthesized in the following manner:

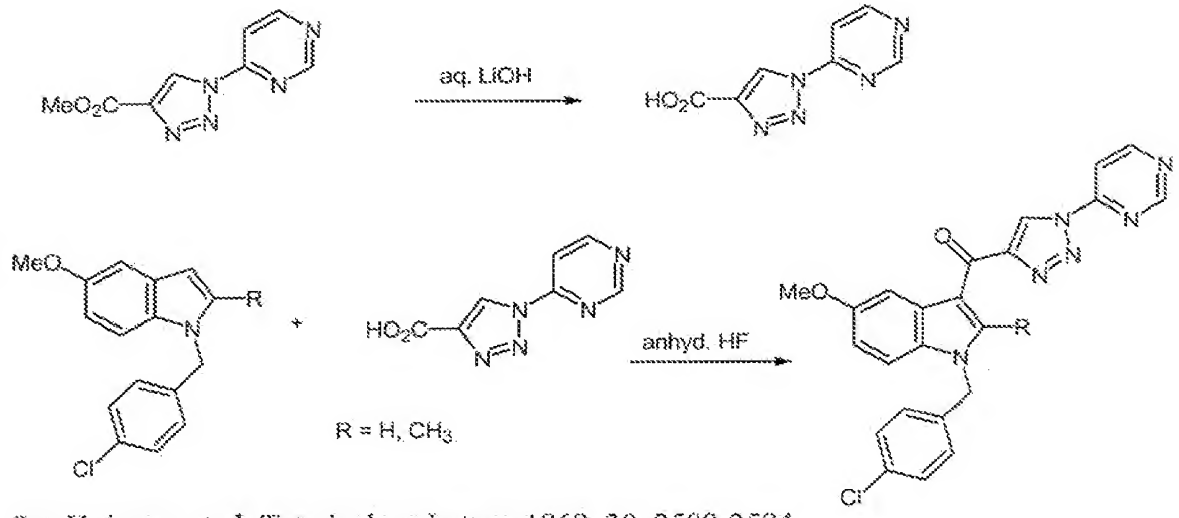


For more information regarding CA Registry no. 10296-29-4 see Dyall et al. Australian Journal of Chemistry 1996, 49, 761-765.



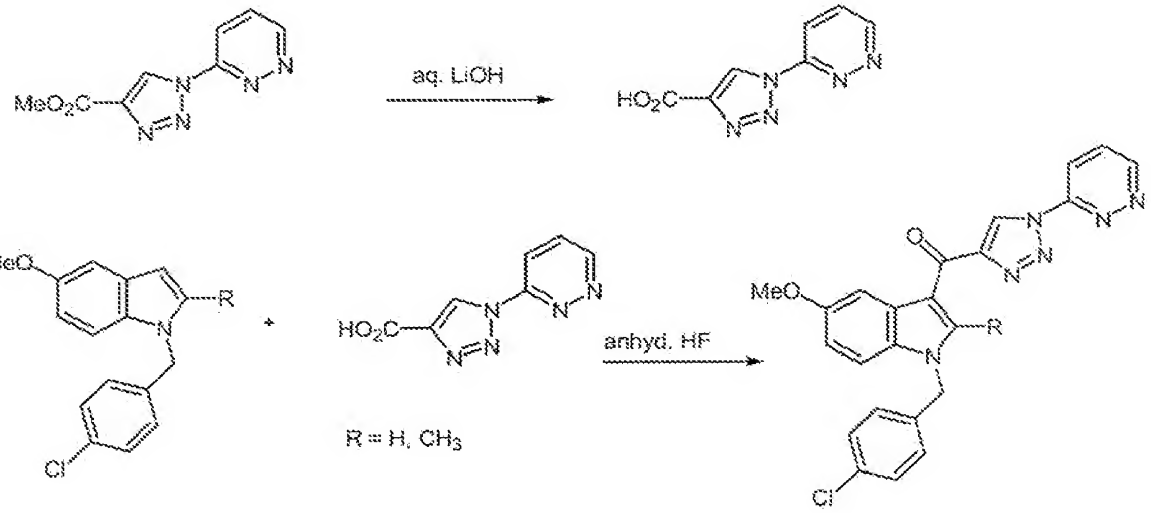
See L'abbe and Beenaerts Tetrahedron 1989, 45, 749-756. The starting material can be obtained using the following reaction:

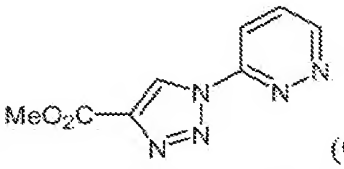




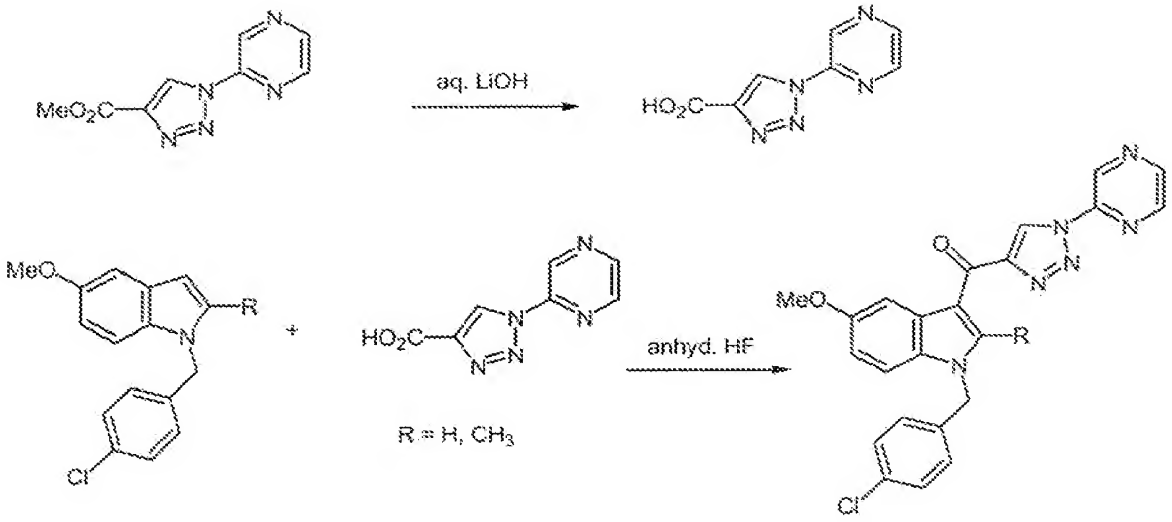
See Huisgen, et al. Tetrahedron Letters 1969, 30, 2589-2594.

The following reaction can be used to obtain the starting material:





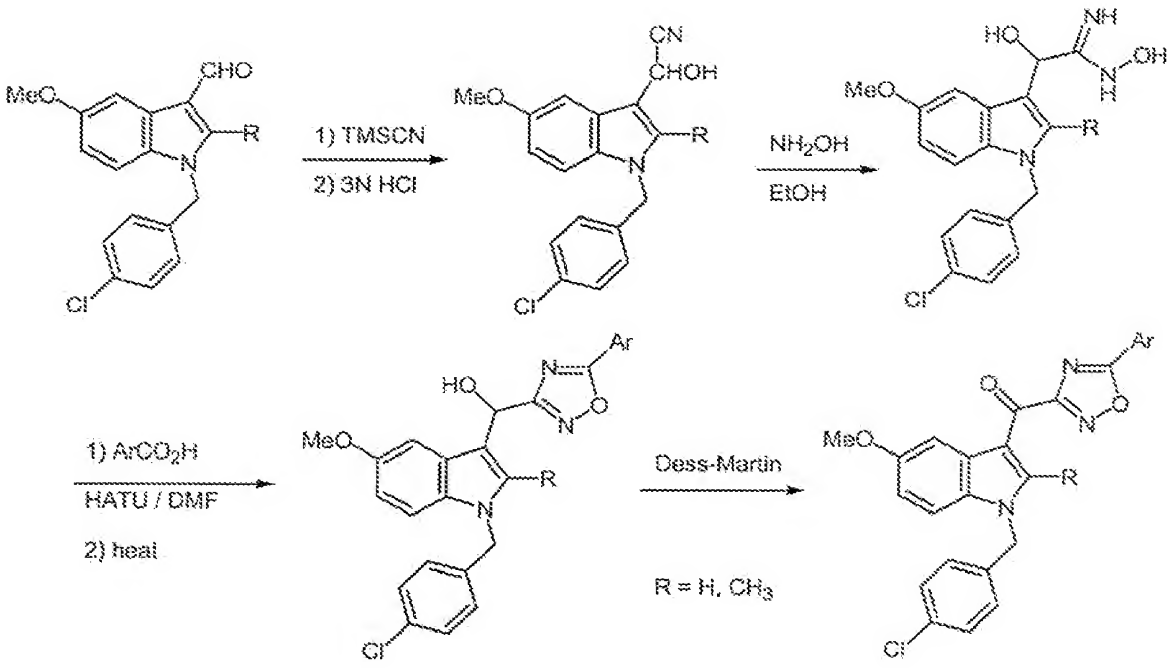
For information regarding the starting material (CA Registry no. 512777-87-6) see Japelj et al. Journal of Heterocyclic Chemistry 2005, 42, 1167-1173.



See Huisgen et al. Tetrahedron Letters 1969, 30, 2589-2594.

The starting material can be obtained by the following reaction:

General method for the preparation of 1,2,4-oxadiazole containing ketone derivatives:



In this schema, HATU is O-(7-Azabenzotriazole-1-yl)-N, N,N'N'-tetramethyluronium hexafluorophosphate (CA Registry no. 148893-10-1), TMSCN is Me_3SiCN (7677-24-9) and Dess-Martin is Periodinane (CA Registry no. 87413-09-0). Also see Lee et al Biorg. Med. Chem. Lett. 2006, 16, 4036-4040.

Other useful syntheses are found in Nazare et al. Angew. Chem. Int. Ed. 2004, 43, 4526 --4528 and patent publication WO9303022 (including the syntheses described in examples 1-428).

Labels

It will be recognized that the compounds of this invention can exist in forms in which one isotope of a particular atom may be replaced with a different isotope of that same atom. For example, "hydrogen" may be ^{1}H, ^{2}H or ^{3}H; "carbon" may be ^{12}C, ^{13}C, or ^{14}C;

"nitrogen" may be ^{14}N or ^{15}N; "oxygen" may be ^{16}O, ^{17}O or ^{18}O; and the like. It will be recognized that the compounds of this invention can exist in radiolabeled form, i.e., the compounds may contain one or more atoms containing an atomic mass or mass number different from the atomic mass or mass number usually found in nature. Radioisotopes of hydrogen, carbon, phosphorous, fluorine, iodine and chlorine include ^{3}H, ^{14}C, ^{35}S, ^{18}F, ^{32}P, ^{33}P, ^{125}I, and ^{36}Cl, respectively. Compounds that contain those radioisotopes and/or other radioisotopes of other atoms are within the scope of this invention. Tritiated, i.e. ^{3}H, and carbon-14, i.e., ^{14}C, radioisotopes are particularly preferred for their ease in preparation and detectability. Radiolabeled compounds described herein and prodrugs thereof can generally be prepared by methods well known to those skilled in the art. Conveniently, such radiolabeled compounds can be prepared by carrying out the procedures disclosed in the Examples and Schemes by substituting a readily available radiolabeled reagent for a non-radiolabeled reagent.

The labels can be primary labels (where the label comprises an element which is detected directly) or secondary labels (where the detected label binds to a primary label, e.g., as is common in immunological labeling). An introduction to labels, labeling procedures and detection of labels is found in Introduction to Immunocytochemistry, (2d ed.) Polak and Van Noorden,, Springer Verlag, N.Y. (1997) and in Handbook of Fluorescent Probes and Research Chemicals, Haugland (1996), a combined handbook and catalogue published by Molecular Probes, Inc., Eugene, Oreg. Primary and secondary labels can include undetected elements as well as detected elements. Useful primary and secondary labels in the present invention can include spectral labels, which include fluorescent labels such as fluorescent dyes (e.g., fluorescein and derivatives such as fluorescein isothiocyanate (FITC) and Oregon Green™, rhodamine and derivatives (e.g., Texas red, tetramethylrhodamine isothiocyanate (TRITC), etc.), digoxigenin, biotin, phycoerythrin, AMCA, CyDyes™ and the like), radiolabels (including those described above), enzymes (e.g., horseradish peroxidase, alkaline phosphatase etc.) spectral colorimetric labels such as colloidal gold or colored glass or plastic (e.g. polystyrene, polypropylene, latex, etc.) beads. The label may be coupled directly or indirectly to a compound described herein according to methods well known in the art. As indicated above, a wide variety of labels

may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions. In general, a detector which monitors a protein/inhibitory agent interaction is adapted to the particular label which is used. Typical detectors include spectrophotometers, phototubes and photodiodes, microscopes, scintillation counters, cameras, film and the like, as well as combinations thereof. Examples of suitable detectors are widely available from a variety of commercial sources known to persons of skill.

Nonlimiting examples of labels include those which utilize 1) chemiluminescence (using horseradish peroxidase or alkaline phosphatase with substrates that produce photons as breakdown products) with kits being available, e.g., from Molecular Probes, Amersham, Boehringer-Mannheim, and Life Technologies/Gibco BRL; 2) color production (using both horseradish peroxidase or alkaline phosphatase with substrates that produce a colored precipitate) (kits available from Life Technologies/Gibco BRL, and Boehringer-Mannheim); 3) fluorescence (e.g., using Cy-5 (Amersham), fluorescein, and other fluorescent tags); and 4) radioactivity. Other methods for labeling and detection will be readily apparent to one skilled in the art.

In one embodiment, the label is a fluorescent label. Fluorescent labels have the advantage of requiring fewer precautions in handling, and being amenable to high-throughput visualization techniques (optical analysis including digitization of the image for analysis in an integrated system comprising a computer). Preferred labels are typically characterized by one or more of the following: high sensitivity, high stability, low background, low environmental sensitivity and high specificity in labeling. Fluorescent moieties, which are incorporated into the labels of the invention, are generally are known, including Texas red, digoxigenin, biotin, 1- and 2-aminonaphthalene, p,p'-diaminostilbenes, pyrenes, quaternary phenanthridine salts, 9-aminoacridines, p,p'-diaminobenzophenone imines, anthracenes, oxacarbocyanine, merocyanine, 3-aminoequilenin, perylene, bis-benzoxazole, bis-p-oxazolyl benzene, 1,2-benzophenazin, retinol, bis-3-aminopyridinium salts, hellebrigenin, tetracycline,

sterophenol, benzimidazolylphenylamine, 2-oxo-3-chromen, indole, xanthen, 7-hydroxycoumarin, phenoxazine, calicylate, strophanthidin, porphyrins, triarylmethanes, flavin and many others. Many fluorescent tags are commercially available from the SIGMA chemical company (St Louis, MO), Molecular Probes, R&D systems (Minneapolis, MN), Pharmacia LKB Biotechnology (Piscataway, N.J.), CLONTECH Laboratories, Inc. (Palo Alto, CA), Chem Genes Corp., Aldrich Chemical Company (Milwaukee, WI), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersberg, MD), Fluka ChemicaBiochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), and Applied Biosystems (Foster City, CA), as well as many other commercial sources known to one of skill.

The labels may be covalently bound to the compounds described herein by a tether group. The tether group can be any moiety capable of covalently linking to the compounds and to the labels. Preferred groups are substituted or unsusbstituted alkylene, alkenylene or alkynylene of 1 to 10 carbon atoms, more preferably 1 to 4 carbon atoms. Particularly preferred groups are unsusbstituted alkynylenes.

METHODS FOR ASSESSING ACTIVITY *IN VITRO* AND *IN VIVO*

COX RELATED ASSAYS

COX-1 and COX-2 Inhibition: Purified Enzyme Assays

The *in vitro* COX-1 and COX-2 inhibitory activity of the compounds described herein can be measured using a test kit available from Cayman Chemical (Ann Arbor, MI). Because COX-1 and COX-2 convert arachidonic acid to prostaglandin H_2 (PGH_2), one can assess COX inhibitory activity of a test compound by measuring the effect of the compound on PGH_2 production in the presence of purified COX-1 enzyme and in the presence of purified COX-2 enzyme. In this assay, the production of PGH_2 can be measured by reducing PGH_2 to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) with $SnCl_2$ and then detecting $PGF_{2\alpha}$ by enzyme immunoassay (EIA) using a suitable antibody. Figure 1, Figure 3

Figure 5, and Figure 6 provide activity data for certain compounds tested for inhibition of Cox-1 and Cox-2 using the Cox-1 and Cox-2 purified enzyme assays.

COX-1 and COX-2 Inhibition: Human Whole Blood Assay

A human whole blood assay can also used to measure the inhibitory activity of each compound on COX-1 and COX-2. Briefly, human whole blood is drawn from 3-6 healthy volunteers who have not taken NSAIDS the previous 2 weeks. To measure COX-1 activity in whole blood, 100 µl of whole blood is combined with a 2 µl aliquot of test compound in vehicle or vehicle alone and incubated for 1 hour at 37 °C as described by Berg et al. (1999 *Inflamm. Res.* 48, 369-379). Serum is isolated from the sample by centrifugation at 12,000g for 5 minutes at 4 °C and then assayed for thromboxane B2 (TXB2) levels using an ELISA assay (e.g., Cayman EIA Kit, Catalog Number 519031). To measure COX-2 activity in whole blood, 100 µl of heparinized whole blood is combined with a 1 µl aliquot of 10 mg/mL LPS (lipopolysaccharide) and a 2 µl aliquot of test compound in vehicle or vehicle alone and incubated for 24 hours at 37 °C as described by Berg et al. (*supra*). Serum is isolated from the sample by centrifugation at 12,000g for 5 minutes at 4 °C and assayed for prostaglandin E_2 (PGE_2) using an ELISA assay (e.g., Cayman EIA Kit, Catalog Number 514010). Figure 3 and Figure 6 provide activity data for certain compounds tested for inhibition of Cox-1 and Cox-2 using the Cox-1 and Cox-2 human whole blood assays.

In the Cox-1 and Cox-2 assays 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]acetic acid (indomethacin), a non-selective Cox inhibitor was used as a control. The Cox-2 selective inhibitors, 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide (Celecoxib), 4-(5-methyl-3-phenylisoxazol-4-yl)benzenesulfonamide (valdecoxib), and 4-[4-(methylsulfonyl)phenyl]-3-phenylfuran-2(5H)-one (rofecoxib) were also used as controls.

FAAH RELATED ASSAYS

FAAH Inhibition: Human Whole Cell Assay and Rat and Human Brain Homogenate Assays

The ability of compounds to inhibit FAAH can be measured in human whole cell and human and rodent brain homogenates as described herein.

FAAH Rat Brain Membrane (RBM) Homogenate Preparation

Adult rats (Charles River CD strain, female, 200 g) are anaesthetized with isofluorane and rapidly decapitated, respectively. Each brain is quickly removed and chilled in tubes (3 brains per tube) on ice. About 25 mL of "homogenization buffer" (20 mM HEPES buffer, pH 7.0, with 1 mM $MgCl_2$) is added to 15 to 20 g of brain. The brains are homogenized on ice for 1 minute using an Omni GLH homogenizer (Omni International, Marietta, Georgia). The homogenates are transferred to three centrifuge tubes and centrifuged at 36,500g for 20 minutes at 4 °C. The supernatant is discarded and each pellet is re-suspended in 25 mL "homogenization buffer". The re-suspended material is again centrifuged (36,500g, 20 minutes at 4 °C). Pellets are combined by resuspension in 10 mL of "homogenization buffer" and incubated in a 37 °C water bath for 15 minutes. The tubes are then placed on ice for 5 minutes followed by centrifugation at 36,500g for 20 minutes at 4 °C. The supernatant is discarded and the membrane pellets are then re-suspended in 40 mL of "resuspension buffer" (50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 3 mM $MgCl_2$). A Bradford Protein assay is performed to determine protein concentration. The protein is aliquotted into screw cap Cryo tubes each containing ~ 400 µL, flash frozen in liquid nitrogen and stored at –80 °C until used for the assay. A similar protocol can be used to obtain brain membrane homogenates from mice.

FAAH Human Brain Membrane (HBM) Homogenate Preparation

About 10 g of frozen normal human brain cortex tissue is obtained (e.g., from Analytical Biological Services (ABS), Inc. (Wilmington, DE)). The brain tissue is thawed and transferred to a large ceramic mortar on ice. 50 mL of ice-cold "homogenization buffer"

(20 mM HEPES buffer, pH 7.0, with 1 mM $MgCl_2$) is added to the mortar and the tissue is homogenized with a pestle. The homogenate is centrifuged at 36,500g for 20 minutes at 4 °C. The supernatants are discarded and the pellets are re-suspended in "homogenization buffer" and centrifuged as before. The supernatants are again discarded and the pellets are re-suspended in 30 mL homogenization buffer and incubated in a 37 °C water bath for 20 minutes. The homogenate is then centrifuged as before. The supernatant is discarded and the membrane pellets are re-suspended in 30 mL "resuspension buffer" (50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 3 mM $MgCl_2$). A Bradford Protein assay is performed to determine protein concentration. The protein is aliquotted into screw cap Cryo tubes each containing ~ 200 µL, flash frozen in liquid nitrogen and stored at –80 °C until used for the assay.

FAAH Human Carcinoma Cell Membrane (HCM) Homogenate Preparation

Human breast epithelial carcinoma MCF7 cells are obtained from the American Type Culture Collection (ATCC Number HTB-22, Manassas, VA) and cultured as essentially as described by ATCC. Briefly, cells are grown in Eagle's Minimum Essential Medium (ATCC catalog no. 30-2003) supplemented with 4 mM L-glutamine, 10% final volume of fetal bovine serum (ATCC catalog no. 30-2020), and 0.1 mg/mL human recombinant insulin (Sigma, St. Louis, MO). The cells are grown in 5% carbon dioxide in air. When cells reach ~80% confluency, adherent cells are rinsed with Hanks Balanced Salts Solution (ATCC catalog no. 30-2213), scraped into suspension and collected by centrifugation in a clinical centrifuge at room temperature. Cell pellets are then washed by resuspension in Hanks Balanced Salts Solution followed by centrifugation. Cell pellets are then flash frozen in a dry ice and ethanol bath and stored at –80 °C. Cell pellets are thawed and 25 mL of homogenization buffer is added. Membrane homogenates of MCF7 cells are then prepared as described above for rat brain homogenates. A Bradford Protein assay is performed to determine the protein concentration. The protein is aliquotted into screw cap Cryo tubes each containing ~ 200 µL, flash frozen in liquid nitrogen and stored at –80 °C until used for the assay.

Determination of FAAH Activity

FAAH activity is assayed in the respective homogenates (Rat brain, Human brain, or Human breast cell carcinoma MCF7 cell) using a modification of the method of Omeir et al. (1995 Life Sci 56:1999) and Fowler et al. (1997 J. Pharmacol Exp Ther 283:729). For assay of FAAH in rat brain membrane homogenates (RBM), RBM homogenates (7 µg protein in 20 µl final volume of 10 mM Tris pH 6.5) are mixed with 180 µl of a mixture of the following: 2.0 µM unlabelled anandamide, 0.03 µCi radiolabeled anandamide [ethanolamine 1-^{3}H] (40-60 Ci/mmol, product number ART-626, American Radiolabelled Chemicals, St. Louis, MO), 1 mg/mL Bovine Serum Albumin (fatty acid-free BSA, electrophoresis grade, Sigma, St. Louis, MO), 10 mM Tris-HCl (pH 6.5), and 1 mM EDTA in the presence and absence of test compounds (vehicle is DMSO at a final concentration of 1%) and incubated for 10 minutes at 37 °C. Samples are placed on ice to terminate the reactions. ^{3}H-ethanolamine product and un-reacted ^{3}H-anandamide substrate are separated by either: (1) using chloroform/ methanol extraction or (2) by passing the reaction mixture through a glass fiber filter containing activated charcoal. Samples are extracted with chloroform/methanol by adding 0.4 mL of chloroform/methanol (1:1 v/v), vigorously mixing the samples, and separating the aqueous and organic phases by centrifugation. Radioactivity (corresponding to FAAH-catalyzed breakdown of ^{3}H-anandamide) found in aliquots (0.2 mL) of the aqueous phase is determined by liquid scintillation counting with quench correction. IC_{50} values are determined as described by Jonsson et al. (2001 Br *J Pharmacol* 133:1263). Alternatively, reactions are purified using a modification of the solid-phase extraction method described by Wilson et al (2003 Anal Biochem 318 : 270). This method can be modified as follows: after reactions are incubated at 37°C for 10 minutes and chilled on ice, the reaction mixtures are acidified by adding 10 µl of sodium phosphate solution [0.5M (pH 2.0)]. 90 µl aliquots of the acidified reaction mixtures are applied to activated charcoal (that has been previously washed with methanol as described by Wilson et al. (supra)) containing 80 µl of water on top of a glass fiber filter, centrifuged, and the radioactivity in the eluate is counted as described previously by Wilson et al. (supra) Figure 3, Figure 5, and Figures 9a, 9b, 9c and 9d provide activity data for certain

compounds tested for inhibition of FAAH using the FAAH rat and human brain homogenate assay. The known FAAH inhibitors, 3'-(aminocarbonyl)biphenyl-3-yl cyclohexylcarbamate (URB597), [1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]acetic acid (indomethacin) and 5-benzoyl-2,3-dihydro-1H-pyrrolizine-1-carboxylic acid (Ketorolac) were used as controls in these assays.

Whole cell anandamide hydrolysis assay

FAAH activity can be assayed in whole cells using methods disclosed previously (Maccarone et al. 1998 *J Biol Chem* 273:32332 and Bisogno et al. 1997 J Biol Chem 272 :3315). In addition to the cell lines described in Maccarone et al. and Bisogno et al., MCF7 (ATCC designation HTB-22) and T84 (ATCC designation CCL-248) cell lines may be used in these assays.

Determination of Endogenous and Exogenous Anandamide Levels in Rat Plasma and Brain Tissue

The effects of test compounds on endogenous and exogenously dosed anandamide (AEA) levels can be measured. Rats dosed with test compound are sacrificed at various time points to determine the levels of anandamide both circulating and within the brain tissue. For experiments measuring exogenous levels of anandamide, the anandamide (Cayman Chemical, Ann Arbor, MI or Sigma Chemical, St. Louis, MO) is dosed (in the range of 3-30 mg/kg) post dosing of test compound. Animals are sacrificed at 5, 15, 30, or 60 minutes after anandamide administration with anesthesia administration followed by decapitation. Brains are removed immediately and the plasma is recovered from the blood for analysis of anandamide levels.

Flash frozen whole brain (e.g. from rat or mouse) samples are first transferred to clean 50-mL conical tubes and the wet brain weight is recorded. Fifteen mL of 9:1 ethyl acetate:hexane and 40 ng of deuterated anandamide (d8AEA) are added to the brain samples. The samples are then homogenized with an Omni GLH homogenizer until the solution is a uniform slurry, and 5.0 mL of water is added just prior to completion. Upon completion of the homogenization the tubes are held on ice. The chilled tubes are then

vortexed and centrifuged at 4 °C at 3500 rpm for 10 minutes. One milliliter of the aqueous layer is sampled for use in a Bradford assay of protein content (Bradford, M.M. *Anal. Biochem.* 1976, 72: 248). The ethyl acetate layer is recovered, placed in a 15-mL glass tube, and evaporated under nitrogen in a TurboVac. Once dry, samples are reconstituted in 1 mL of 1:3 (v/v) $CHCl_3$:methanol and vortexed. Prepared brain samples are transferred to a 96-well plate for analysis by LC/MS/MS.

Stock standards are prepared at 0.0, 0.50, 1.0, 5.0, 10.0, 50.0, 100, 500, and 1000 ng/mL in methanol. Standards for LC/MS/MS are prepared with 0.5 mg Pefabloc, 10 µL of the stock standard to 90 µL of stock rat plasma and vortexing.

Frozen plasma samples containing pefabloc are thawed, and 100 µL of each sample is transferred to a microcentrifuge tube. To each standard and sample tube, 20 ng d8AEA and 100 µL of ice cold acetone (for protein precipitation) is added. Tubes are vortexed, and centrifuged at 13,000 rpm for at least 5 minutes. The supernatants are collected in microcentrifuge tubes and the acetone is evaporated off in a TurboVac for 5-10 minutes. The evaporated supernatant solutions are next extracted with 250 µL of 1:2 (v/v) methanol:$CHCl_3$ and centrifuged at 13,000 rpm for at least 5 minutes. The $CHCl_3$ layer is collected and evaporated under nitrogen (TurboVac) until dry. Standards and samples are then reconstituted in 200 µL of 1:3 (v/v) $CHCl_3$:methanol. Prepared standards and plasma samples are transferred to a 96-well plate for analysis by LC/MS/MS. Similar experiments can be performed using human plasma to which test compounds and exogenous anandamide is dosed or not dosed.

The LC/MS/MS method uses a Waters 2777 sample manager, 1525 binary pump, and Quattro micro mass spectrometer. The separation is performed on a Waters Xterra MS C8, 5 µm, 2.1 x 20 mm analytical LC column with a Thermo Electron Javelin Basic 8, 2 x 10 mm guard column at a flow rate of 0.30 mL/minutes and a 25-µL injection volume. A binary linear gradient of mobile phase A (10 mM ammonium acetate in water (pH 9.5)) and mobile phase B (80:20 acetonitrile:methanol) is used from 2.0 to 2.2 minutes from 25% to 90% B, with a total run time of 6.0 minutes per sample injection. AEA and d8-AEA elute in ~ 3.5 minutes. The Quattro micro is operated in multiple reaction monitoring (MRM) mode with negative electrospray ionization. The mass transitions of 348 m/z - 62 m/z (AEA) and 356 m/z - 62 m/z (d8-AEA) are monitored

using optimized collision settings (determined experimentally). Data are analyzed using Micromass QuanLynx software, and standard curves are generated using the ratio of the internal standard (d8-AEA) peak area to AEA peak area in response to AEA concentration. AEA concentration in brain and plasma samples is calculated using the linear regression of the standard curve. AEA concentration in plasma is reported as ng AEA/mL plasma, and AEA concentration in brain is reported as ng AEA/g protein (protein content determined by the Bradford assay).

Arachidonyl 7-amino, 4-methyl coumarin amide (AAMCA) based FAAH activity assay

The ability of certain compounds described herein to inhibit the ability of FAAH to catalyze the hydrolysis of AAMCA (a fluorogenic substrate) to generate arachidonic acid and a highly fluorescent 7-amino, 4-methyl coumarin (AMC) was determined using essentially the assay described in Ramarao et al. 2005 Anal Biochem. 343:143-151. Figure 9a provides activity data for certain compounds tested in the AAMCA assay.

Determination of Exogenous [^{3}H]anandamide Levels in Mouse blood and Brain Tissue

The effects of test compounds on exogenously dosed [^{3}H]anandamide (including metabolites thereof) levels and localization can be measured as described in Glaser et al 2005 J Pharmacol Exp Ther. Nov 8; 2005.

Measurement of rectal temperature in mice and rats.

Direct acting cannabinoid type 1 (CB1) receptor agonists, including the endo-cannabinoid anandamide, have been shown to produce a rapid decrease in body temperature (hypothermia) following administration to both mice and rats. Test compounds can be tested in such an assay by assessing their ability to potentiate the hypothermic effects of intravenously (IV) dosed anandamide for example as described in Smith et al. (1994) Pharmacol Exp Ther. 270:219-27. Briefly, mice or rats may be administered vehicle or various doses of test compounds. Then, following a suitable pretreatment time, core body temperature can be measured and recorded using a suitably

sized temperature probe inserted in to the anus and rectum. Once baseline temperature is recorded, mice or rats may be administered an IV dose of the endo-cannabinoid anandamide. Subsequently, core body temperature is measured and recorded at various time points until it returns to at or around baseline.

MAGL related assays

Compounds can be tested for their ability to modulate (e.g. inhibit) MAGL activity using the assay described in Ghafouri et al. 2004 Br J Pharmacol 143:774-84. Briefly, cerebella previously obtained from adult Sprague–Dawley rats are thawed and homogenized at 41°C in sodium phosphate buffer (50mM, pH 8) containing 0.32M sucrose. Homogenates are centrifuged at 100,000g for 60 minutes at 41°C. The supernatants ('cytosol fractions') are collected and the pellets are suspended in sodium phosphate buffer (50mM, pH 8) ('membrane fractions'). The samples are stored frozen in aliquots at -70°C until used for the assay. Protein concentration is determined by using the method described by Ghafouri et al. The assay can also be performed using the rat or human brain membrane homogenates whose preparation is described herein. Hydrolysis rates of ^{3}H-2-AG or ^{3}H-2-oleoylglycerol (2-OG, a shorter homologue of 2-AG) are determined as described by Dinh et al. (2002) Proc. Natl. Acad. Sci. U.S.A., 99, 10819–10824. Briefly, membrane or cytosol fractions are diluted to the appropriate assay protein concentrations in tris-HCl buffer (10mM, pH 7.2) containing 1mM EDTA, unless otherwise stated. Aliquots (165 ml) are then added to glass tubes in the presence or absence 10 ml of test compound or vehicle only. Blanks contain assay buffer instead of homogenate solution. Substrate (25 ml, final concentration 2 mM unless otherwise stated) is then added and the samples are incubated for 10 minutes at 37°C. Reactions are stopped by adding 400 µl chloroform:methanol (1/1 v/v^{-1}), vortex mixing the tubes two times and placing them on ice. The phases are then separated by centrifugation (10 minutes, 2500 rpm). Aliquots (200 ml) of the methanol/buffer phase are taken and measured for tritium content by liquid scintillation spectroscopy with quench correction. Alternatively, the ability test compounds to modulate (e.g. inhibit) MAGL activity can be

determined using the assay described in Saario et al. 2004 Biochem Pharmacol 67:1381-7. Rat cerebellar membranes preparations as described by Saario et al. or the rat or human brain membrane homogenates whose preparation is described herein are tested. Briefly, the experiments are carried out with preincubations (80 mL, 30 minutes at 25°C) containing 10 mg membrane protein/membrane homogenate preparation, 44 mM tris–HCl (pH 7.4), 0.9 mMEDTA, 0.5% BSA and 1.25% (v/v) DMSO as a solvent for test compounds. The preincubated membranes are kept at 0°C just prior to the experiments. The incubations (90 minutes at 25°C) are initiated by adding 40 µL of preincubated membrane cocktail, giving a final volume of 400 µL. The final volume contains 5 mg membrane protein/ membrane homogenate preparation, 54 mM Tris–HCl (pH 7.4), 1.1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5% BSA, and 50 mM of the substrate (2-AG, 1(3)-AG, or noladin ether (an ether linked analog of 2-AG)). At time-points of 0 and 90 minutes, 100 µL samples are removed from incubation, acetonitrile (200 µL) is added to stop the enzymatic reaction, and the pH of the samples is simultaneously decreased with phosphoric acid (added to acetonitrile) to 3.0, in order to stabilize 2-AG against a possible post-incubation chemical acyl migration reaction to 1(3)-AG. Samples are centrifuged at 23,700 g for 4 minutes at room temperature prior to HPLC analysis of the supernatant. The specific enzyme activity towards 2-AG or 1(3)-AG is determined as described. Alternatively, the terminated reactions from either the Ghafouri et al. or Saario et al. protocols can be assayed by purification using a modification of the solid-phase extraction method described by Wilson et al (2003 Anal Biochem 318 : 270). 90 µl of the terminated reaction mixtures are applied to activated charcoal (that has been previously washed with methanol as described by Wilson et al.) containing 80 µl of water on top of a glass fiber filter, centrifuged, and the radioactivity in the eluate is counted as described previously by Wilson et al. Inhibition of MAGL activity is measured by the formation of the hydrolysis product ^{3}H-glycerol.

CRTH2 RELATED ASSAYS

CRTH2 agonist assay

CRTH2 agonists increase the expression of CD11b on eosinophils. Neutrophils do not express CRTH2. They do, however, express receptors for other lipid mediators, including 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE), leukotriene B4 (LTB4), and platelet activating factor (PAF). Therefore, any increased expression of CD11b by neutrophils is likely to be caused by an activity other than activation of CRTH2. Accordingly, preferred CRTH2 agonist compounds increase CD11b expression on eosinophils, but not on neutrophils.

The potential CRTH2 agonist activity of certain compounds was tested using a CD11b expression assay using essentially the method described by Monneret et al. (*J Pharmacol Exp Ther* 304:349-55, 2003), and the results of this analysis are presented in Figure 2a Figure 3, and Figure 7 where the results of one or more experiments are reported.

Briefly, polymorphonuclear cells (0.5 ml; 10^6/ml cells) in PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$) were incubated with a test compound at room temperature for 10 minutes. The incubations were terminated by the addition of ice-cold FACSFlow (BD Biosciences; Cat# 342003) and centrifugation (400 g for 5 minutes at 4°C). The cells were then incubated for 30 minutes at 4°C with a mixture of PE-labeled mouse anti-human VLA-4 (5 µl; BD Biosciences) and FITC-labeled mouse anti-human CD11b (10 µl; Beckman Coulter). The cells were then incubated with Optilyse C (0.25 ml; Beckman Coulter) for 15 minutes, centrifuged, and then fixed in PBS (0.4 ml; calcium and magnesium free) containing 1% formaldehyde. The distribution of fluorescence intensities among 60,000 cells was measured by flow cytometry. Eosinophils were gated out on the basis of their granularity (high side scatter) and labeling with VLA-4 (PE fluorescence). CD11b was then measured in the eosinophil region on the basis of fluorescence due to FITC. All data were corrected for the value obtained for the corresponding isotope control antibody.

The results presented in Figure 2a and Figure 7 are reported as the percentage of CD11b expression as compared to the maximum response generated by the positive control 15R-methyl PGD_2 ((5E,9α,13E,15R)-9,15-dihydroxy-15-methyl-11-oxoprosta-5,13-dien-1-oic acid). Compounds with greater than 30% CD11b activity in this assay were considered to be CRTH2 agonists. Figure 3 and Figure 7 provide EC_{50} data for certain compounds tested using this CRTH2 assay.

To confirm that the CD11b expression is caused by activation of the CRTH2 receptor certain controls were performed. Accordingly, effect of the compounds on CD11b expression in neutrophils was tested. If the compound increases CD11b expression in neutrophils, the mobilization in eosinophils is likely caused by an activity other than activation of the CRTH2 receptor. In all cases tested CD11b expression was only observed in eosinophils.

CRTH2 antagonist assay

The potential CRTH2 antagonist activity of certain compounds was tested using an assay that tests the ability of the compounds to block the CD11b expression in eosinophils by 15-R-methyl-PGD_2 using essentially the method described above for the agonist assay except that the cells were preincubated with various concentrations of compounds before they were challenged with the agonist 15R-Methyl-PGD_2. The results of this analysis are presented in Figure 2b. A CRTH2 antagonist should block CD11b expression by subsequently added 15-Methyl-PGD_2. The results presented in Figure 2b are reported as percentage of inhibition of the maximum response generated by 15R-Methyl-PGD_2. Ramatroban (3-((3R)-3-{[(4-fluorophenyl)sulfonyl]amino}-1,2,3,4-tetrahydro-9H-carbazol-9-yl)propanoic acid) and [1-(1,3-benzothiazol-2-ylmethyl)-5-fluoro-2-methyl-1H-indol-3-yl]acetic acid both known CRTH2 antagonists were used as positive controls in this assay. Compounds with 85% or greater inhibition in this assay are considered to be CRTH2 antagonists. The assay can be also performed in the presence of human plasma which is added to a final concentration of 10% in the initial incubation with PBS buffer. Figure 3 and Figure 7 provide % inhibition of CRTH2 activity and IC_{50} data for certain compounds using this assay.

Alternatively, CRTH2 antagonist activity can be determined by a calcium mobilization assay conducted as follows, adapted from the protocol described by Monneret et al. (*J Pharmacol Exp Ther* 304:349-55, 2003). Briefly, leukocytes (10^7 cells/ml) are treated with the acetoxymethyl ester of fluo-3 (2 μM; Molecular Probes, Eugene, Oregon) in the presence of Pluronic F-127 (0.02%; Molecular Probes) for 60 minutes at 23°C. The mixture is centrifuged at 200 x g for 10 minutes and the pellet resuspended in PBS to give a concentration of 5 x 10^6 cells/ml. The leukocytes are treated with PC5-labeled mouse anti-human CD16 (3.3 μl/10^6 cells; Beckman-Coulter) for 30 minutes at 0°C. PBS (25 ml) is added, the mixture centrifuged as described above, and the pellet resuspended in PBS to give a concentration of 3x10^6 leukocytes/ml. After incubation at 23°C for 30 minutes, an aliquot (0.95 ml) of the leukocyte suspension is removed and treated with PBS (50 μl) containing Ca^{++} (36 mM) and Mg^{++} (20 mM). After 5 minutes, the cells are analyzed by flow cytometry using a FACS Calibur instrument (Becton-Dickinson, San Jose, CA). A total of approximately 10^6 cells are counted over a period of 3 to 4 minutes for each sample. Fluo-3 fluorescence is measured in eosinophils, neutrophils, and monocytes, which are gated out on the basis of CD16 staining and side scatter. Test compounds are added 2 minutes after the start of each run followed 2 minutes later by 15R-Methyl-PGD_2. Maximal calcium responses are determined by addition of the calcium ionophore, A23187 (10 μM) one minute after the addition of 15R-Methyl-PGD_2.

DP-1 RECEPTOR ASSAYS

DP-1 Receptor Antagonist Assay

Human blood is collected in citrate vacutainer tubes. Platelets are isolated at 1-5 x10^7 cells/mL in PBS. Isobutylmethylxanthine (Sigma catalog # 15879) is added to the platelets for a final concentration of 1 mM and 300 μL of platelets are then aliquoted into the appropriate wells of a 96-well assay plate. Samples are then incubated at 37°C for 8 minutes. Next, 3 μL of test compound are added to the appropriate wells of the assay

plate for a final concentration of 10 μM. To create a standard curve, 3 μL of the appropriate concentration of BW-A868C (Cayman chemical catalog # 12060) is added to one row of the assay plate. The standard curve is prepared in dimethylsulfoxide starting at 10 μM and diluting ten-fold to 0.1 nM. Samples are then incubated at 37°C for 10 minutes. 3 μL of the agonist control, BW-245C (Cayman Chemical catalog # 12050), are added to each sample and the samples are incubated for 10 minutes at 37°C. After the 10 minute incubation, 1 mL of ice cold ethanol is added to each sample. Samples are spun for 10 minutes at 600xg and 4°C. 200 μL of supernatant are removed and diluted 1:10 in EIA buffer (provided in Cayman Chemical c-AMP EIA kit-catalog # 581002). A standard curve of cAMP is also prepared in EIA buffer starting at 3000 pmol/mL, diluted two-fold, to 28 pmol/mL. 50 μL of diluted sample are added to the appropriate wells of the ELISA 96-well plate coated with mouse anti-rabbit IgG. 50 μL of the prepared standard curve are also transferred to the ELISA plate. 50 μL of cyclic AMP AChE tracer, reconstituted in 6 mL of EIA buffer, are added to each sample, including the standard curve. 50 μL of cyclic AMP EIA antiserum, reconstituted in 6 mL of EIA buffer, are added to each sample, including the standard curve. Samples are incubated at 4°C for 16-18 hours. After the overnight incubation, samples are dispensed from the plate. The plate is washed five times with wash buffer provided in the cyclic AMP EIA kit. 20 mL of ultrapure water are added to the vial of Ellman's reagent provided in the cyclic AMP kit. 200 μL of reconstituted Ellman's reagent are added to each sample well. Samples are incubated for 90 minutes at room temperature while being protected from light. After 90 minutes, the sample plate is read on a fluoremeter set to 412 nm and endpoint mode. Figure 3 and Figure 8 provide the results of a DP-1 antagonist assay for a number of compounds. The results are presented as a percentage of the maximal response elicited by the known antagonist BW-A868C.

DP-1 Receptor Agonist Assay

Human blood is collected in citrate vacutainer tubes. Platelets are isolated at 1-5 $x10^7$ cells/mL in PBS. Isobutylmethylxanthine (Sigma catalog # 15879) is added to the platelets for a final concentration of 1 mM. 300 μL of platelets are then aliquoted into the

appropriate wells of a 96-well assay plate. Samples are then incubated at 37°C for 8 minutes. 3 µL of test compound or agonist control are added to the appropriate wells of the assay plate for a final concentration of 10 µM. The agonist control used is BW-245C (Cayman Chemical catalog # 12050). Samples are then incubated at 37°C for 10 minutes. After the 10 minute incubation, 1 mL of ice cold ethanol is added to each sample. Samples are spun for 10 minutes at 600xg and 4°C. 200 µL of supernatant are removed and diluted 1:10 in EIA buffer (provided in Caymen Chemical c-AMP EIA kit-catalog # 581002). A standard curve of cAMP is also prepared in EIA buffer starting at 3000 pmol/mL, diluted two-fold, to 28 pmol/mL. 50 µL of diluted sample are added to the appropriate wells of the ELISA 96-well plate coated with mouse anti-rabbit IgG. 50 µL of the prepared standard curve are also transferred to the ELISA plate. 50 µL of cyclic AMP AChE tracer, reconstituted in 6 mL of EIA buffer, are added to each sample, including the standard curve. 50 µL of cyclic AMP EIA antiserum, reconstituted in 6 mL of EIA buffer, are added to each sample, including the standard curve. Samples are incubated at 4°C for 16-18 hours. After the overnight incubation, samples are dispensed from the plate. The plate is washed five times with wash buffer provided in the cyclic AMP EIA kit. 20 mL of ultrapure water are added to the vial of Ellman's reagent provided in the cyclic AMP kit. 200 µL of reconstituted Ellman's reagent are added to each sample well. Samples are incubated for 90 minutes at room temperature while being protected from light. After 90 minutes, the sample plate is read on a fluoremeter set to 412 nm and endpoint mode. Figure 3 and Figure 8 provide the results of a DP-1 agonist assay for a number of compounds. The results are presented as a percentage of the maximal response elicited by the known agonist BW-245C.

Thromboxane A2 (TXA_2) related assays

TXA_2 receptor binding assay

Ramatroban, a known CRTH2 antagonist, is also a thromboxane A2 receptor antagonist. Certain compounds described herein were tested for their ability to bind to the TXA_2 receptor using an assay similar to that described in Schrör et al. 1995 Biochemical Pharmacol 49:921-927. Purified TXA_2 receptors from human platelets (0.6

to 0.8 μg protein/mL) were thawed on ice and preincubated for 1 hour at 4°C in pH 7.4 or pH 6.0 buffer. The incubation mixture (200 μl final volume) consisted of HEPES (25 mM), EDTA (2 mM), CHAPs buffer (10 mM Tris·HCl, pH 8.0/1 mM $MgCl_2$/1 mM EGTA/0.5% CHAPS/10% glycerol/5 mM 2-mercaptoethanol/1 mM DTT) (5 mM), asolectin (0.5 mg/mL), 60-80 ng purified TXA_2 receptor, vehicle or test compounds at various concentrations, 50,000-70,000 cpm [^{125}I]BOP (1S-(1 α,2 β(5Z),3 α(1E, 3R*),4 α)]-7-[3-(3-hydroxy-4-(4'-iodophenoxy)-1-butenyl)7-oxabicyclo- [2.2.1]heptan-2-yl]-5-heptenoic acid; a TXA_2 mimetic) and various concentrations of I-BOP (0.05 to 250 nM) and was incubated for 1 hour at 30°C. Assays were performed in silanized (12 x 75 mm) glass tubes. The mixture was then filtered rapidly through Whatman GF/C glass-fiber filters, presoaked with 0.3 % polyethylenimine. This was followed by three additional washings with ice-cold HEPES (25 mM)/EDTA (2 mM)/CHAP (0.1 mM). The filtration procedure was complete within 10 seconds. Radioactivity was counted using an LKB gamma-counter. Nonspecific binding was defined as the amount of radioactivity bound in the presence of 10 μM L 657925 (a TXA_2 receptor antagonist; Warner et al. (1997) Prostaglandins. 54:581-99). Figure 10 provides TXA_2 receptor binding assay data for certain compounds described herein as measured by % displacement of I-BOP binding. Ramatroban, a known TXA_2 was used as a positive control in this assay.

Bleeding time assay

TXA_2 binds to the TXA_2 receptor to induce platelet aggregation and hemostasis. Test compounds are evaluated for their ability to modify bleeding time in an in vivo assay. Test compounds or vehicle alone are administered orally. The measurement of coagulation time in rats and mice is performed as a terminal study under general anesthesia. Animals are anesthetized and then positioned horizontally on a platform with their tails taped downwards such that the tail is perpendicular to their body and hangs about 2 cm from the top of a platform. The distal portion of the tail is amputated with a scalpel. The amount of bleeding from the transection is measured in one of four ways: 1) Clotting Time in Water: the tail is immersed into water or saline warmed to 37°C and the time to clotting is recorded; 2) Clotting Time in Air: Whatman filter paper is applied to the edge of the forming clot every 30 seconds, taking care not to dislodge the clot.

Blood that continues to flow from the cut during the 30-second interval is allowed to fall on the filter paper at the same point. The amount of time until clotting is recorded; 3) Volume Measurement: blood is collected in a citrated Eppendorf tube for a duration of 5 minutes after the transection. The total volume of whole blood wis measured; and 4) Absorbance Reading: the tail is immersed for 10 minutes in 1 mL of 0.9% NaCl warmed to 37°C. Blood loss is determined by measuring the absorbance of saline at 560 nm and the result is compared to a standard curve constructed from known volumes of mouse blood.

D-AMINO ACID OXIDASE RELATED ASSAYS

Inhibition of porcine kidney DAO

Porcine kidney D-amino acid oxidase (catalog # A-5222 from Sigma) and D-serine (catalog # S-4250 from Sigma) was used to test the DAO inhibitory activity of test compounds. The breakdown of D-serine by DAO produces hydrogen peroxidase, which can be measured using, for example, the Amplex® Red Hydrogen Peroxide Assay Kit (Catalog # A-22188, Molecular Probes, Inc.; Eugene, OR). A working solution was prepared by mixing: distilled water (7.93 mL), sodium phosphate buffer (1 ml, 0.25M, pH 7.4), D-serine solution (1.0 ml, 100 mM in water), horseradish peroxidase (0.02 ml, 100 U/ml in buffer), and Amplex Red solution (0.05 ml, 1 mg dye in 200 ul in DMSO (50 μM in DMSO)). A working enzyme solution is prepared by diluting a D-amino acid oxidase stock solution (65 U/ml) four hundred fold. The working solution (99 μl) was transferred to wells of a Microfluor microtiter plate and a solution of the inhibitor in DMSO (1 μL) is added. The working enzyme solution (20μl) was added to each well and the rate of reaction (hydrogen peroxide released) was determined by measuring the oxidation of Amplex Red by spectrophotometry, using a plate reader (excitation wavelength 544 nm, emission wavelength, 590 nM) after a reaction time of 15 minutes. Controls were carried out using DMSO in the absence of inhibitor. A known DAO inhibitor, indole-2-carboxylic acid, was used as a control in this assay. Figure 3, Figure 5 and Figure 8 present the results of the analysis of certain compounds in the DAO assay.

Inhibition of human DAO

Human D-amino acid oxidase extracts were prepared by harvesting HEK293 cells either transiently or stably transfected with the human DAO clone (huDAO). The stable huDAO cell line was generated by co-transfecting the huDAO gene (Catalog#TC118941, Origene, Rockville, MD) along with pcDNA3.1 (Invitrogen, Carlsbad, CA) at a 100:1 ratio into HEK293 cells under G418 selection. Transient huDAO transfections were implemented using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and following the manufacturers protocol with the following specifics. HEK293 cells were seeded at 2 x 107 cells per T150 flask the day before transfection. huDAO DNA (Catalog#TC118941, Origene, Rockville, MD) was transfected at 37.5 ug per flask and at a 3:1 DNA/Lipofectamine ratio. The DNA/Lipofectamine mixture was incubated on the cells for 48 hrs before cell harvesting. Similar results were obtained with transiently vs stably expressed huDAO. Extracts were harvested as follows. Culture liquid was removed from flasks and replaced with Hank's Buffered Saline Solution (20 mLs). The cells were scraped into the Hank's Buffer and then transferred to a fresh tube. Samples were spun for 10 minutes at 3,000rpm. The supernatant was decanted and the pellet resuspended in 50 mM Tris-HCL pH8.7, 1 μM FAD and 1 mM DTT, 20% glycerol (1 mL). Samples were then homogenized on ice for 20 seconds. Homogenates were spun down for 5 minutes at 3,000rpm. The supernatants were removed and set aside. The pellets were resuspended in 50mM Tris-HCL pH8.7, 1 μM FAD, 1 mM DTT and 0.1% octyl-β-D-glucoside, 20% glycerol (1 mL) and homogenized on ice for 20 seconds. Homogenates were spun for 5 minutes at 3,000rpm. The supernatants were collected and combined with previously collected supernatants for a master stock. Extracts were then serially diluted and tested in the D-amino acid oxidase enzyme assay to determine activity based on protein concentration. Stocks were prepared accordingly, typically, for a twenty fold dilution in future assays.

Human D-amino acid oxidase (HEK293 cells stably transfected with huDAO clone) and D-serine (catalog # S-4250 from Sigma) were used to test the DAO inhibitory activity of test compounds. The breakdown of D-serine by DAO produces hydrogen peroxidase, which can be measured using, for example, the Amplex® Red Hydrogen Peroxide Assay Kit (Catalog # A-22188, Molecular Probes, Inc.; Eugene, OR). A working solution was prepared by mixing: distilled water (7.93 mL), sodium phosphate buffer (1 ml, 0. 25M,

pH 7.4), D-serine solution (1.0 ml, 100 mM in water), horseradish peroxidase (0.02 ml, 100 U/ml in buffer), and Amplex Red solution (0.05 ml, 1 mg dye in 200 ul in DMSO (50 μM in DMSO)). A working enzyme solution was typically prepared by diluting a D-amino acid oxidase stock solution twenty fold. The working solution (99 μl) was transferred to wells of a Microfluor microtiter plate and a solution of the inhibitor in DMSO (1 μL) is added. The working enzyme solution (20μl) was added to each well and the rate of reaction (hydrogen peroxide released) was determined by measuring the oxidation of Amplex Red by spectrophotometry, using a plate reader (excitation wavelength 544 nm, emission wavelength, 590 nM) after a reaction time of 15 minutes. Controls were carried out using DMSO in the absence of inhibitor. A known DAO inhibitor, indole-2-carboxylic acid, was used as a control in this assay. Figure 8B presents the results of the analysis of certain compounds in the DAO assay.

DAO Whole Cell Assay 1 – Toxicity

Human D-amino acid oxidase (huDAO) and D-serine (catalog # S-4250 from Sigma) are used to test the DAO inhibitory activity of test compounds. A stable hDAO cell line is generated by co-transfecting the huDAO gene (Catalog #TC118941 , Origene, Rockville, MD) along with pcDNA3.1 (Invitrogen, Carlsbad, CA) at a 100:1 ratio into HEK293 cells under G418 selection. The intracellular breakdown of D-serine by DAO produces hydrogen peroxide, which induces toxicity to the cell monolayer. This toxicity is measured by, for example, the AlamarBlue™ Reagent (Catalog # BUF012B, AbD Serotec Ltd., Kidlington, Oxford, UK). On day 1 of the assay, the following additions are made, in order, to a black, clear bottom, tissue culture treated 96-well plate (Corning # 3904): 2 ul inhibitor (100x in 100% DMSO, or vehicle), 100 ul 70 mM D-serine in HEK media (DMEM/10%FBS), 100 ul huDAO cells (2 x 10^5/ml). The cells are incubated for 18-24 hrs at 37°C/5%CO2. On day 2 of the assay, 20 ul of AlamarBlue™ Reagent is added to each well, and the plate is returned to the incubator for another 24 hrs. On day 3 of the assay, the amount of cellular toxicity (induced by hydrogen peroxide produced by intracellular huDAO) is determined by measuring the conversion of AlamarBlue reagent in a fluorescent plate reader (excitation wavelength 545 nm, emission wavelength, 590 nM; @ 37°C).

DAO Whole Cell Assay 2 – Amplex Red

Human D-amino acid oxidase (huDAO) and D-serine (catalog # S-4250 from Sigma) are used to test the DAO inhibitory activity of test compounds. A stable huDAO cell line is created by co-transfecting the huDAO gene (Catalog #TC118941 , Origene, Rockville, MD) along with pcDNA3.1 (Invitrogen, Carlsbad, CA) into HEK293 cells under G418 selection. The intracellular breakdown of D-serine by huDAO produces hydrogen peroxide, which is measured by, for example, the Amplex® Red Hydrogen Peroxide Assay Kit (Catalog # A-22188, Molecular Probes, Inc.; Eugene, OR). The following additions are made, in order, to a black, clear bottom, tissue culture treated 96-well plate (Corning # 3904): 2 ul inhibitor (100x in 100% DMSO, or vehicle), 100 ul Detection Solution (30 mM D-serine, 20 uM Amplex Red, 0.05 U/ml HRP in Hanks Balanced Salt Solution/20mM HEPES 7.4), and 100 ul huDAO cells (6 x 10^5/ml). The intracellular huDAO activity is proportional to the rate of hydrogen peroxide produced by the cells and is determined by measuring the conversion of Amplex Red in a fluorescent plate reader (excitation wavelength 544 nm, emission wavelength, 590 nM) at 37°C over a 60 min kinetic read.

Detection of D-amino acids in serum and urine

Serum and urine samples are obtained and immediately frozen in a –80°C freezer before analysis. Serum and urine levels of D-amino acids (aspartate, glutamate, glycine, D-serine, L-serine) are determined by precolumn derivatization with N-tert, -butyloxy-carbonyl-L-cycteine and o-phthaldialdehyde (Hashimoto et al. J Chromatogr (1992) 52:325-53) coupled with a mobile phase gradient of methanol and 100 mmol/L, pH 7.2 sodium acetate, and reverse phase C-18 column for high-pressure liquid chromatography separation with fluorescent detection at excitation wavelength of 433 nm and emission wavelength of 344 nm. The absolute concentrations of amino acids are determined by computer analysis (Maxima 820, Waters, MA) of peak height with internal and external standards. D-amino acid levels (e.g. D-serine) can be determined in the presence and absence of test compound.

Detection of D-amino acids in brain and plasma

Brain and plasma samples are obtained and immediately frozen in a -80°C freezer before analysis. Amino acids were extracted from plasma using a protein precipitation procedure while brains were homogenized under acidic conditions. Levels of D-amino acids (serine, alanine, leucine and proline) are determined by precolumn derivatization with Marfey's reagent (Fluoro-dinitrophenyl-L-alanine amide) (Berna M.J. and Aekermann B.L. (2006) J Chromatogr B; doi:10.1016/j.jchromb.2006.08.029) coupled with a mobile phase gradient of 15 mM ammonium acetate in a combination of water, methanol and acetonitrile on a reverse phase C-18 column for high-pressure liquid chromatography separation with mass spectrometry detection in the negative single ion reaction mode. The absolute concentrations of amino acids are determined by computer peak area ratio with internal standards. D-amino acid levels (e.g. D-serine) can be determined in the presence and absence of test compound.

D-serine induced nephrotoxicity

D-serine and D-propargylglycine have been associated with nephrotoxicity and induce one or more of glucosuria, aminoaciduria, proteinuria, and polyuria. Compounds which inhibit DAO activity may also control the production of toxic metabolites of D-amino acid oxidation (e.g. D-serine) such as hydrogen peroxide and ammonia. Hydrogen peroxide and concomitantly produced oxygen radicals may lead to nephrotoxicity. Compounds described herein can be evaluated for their ability to attenuate the nephrotoxicity associated with D-serine or D-propargylglycine administration in rats as described in Williams and Lock 2005 Toxicology: 207:35-48 and Maekawa et al. 2005 Chem Res Toxicol. 18:1678-1682.

Measurements of NMDA Receptor Affinity

To measure the affinity of the compounds reported herein for D-serine's binding site on the NMDA receptor (also known as the "Glycine site" or the "strychnine-insensitive glycine site"), a radioligand-binding assay is performed with membranes prepared from rat cerebral cortex. The radioactive ligand is [^{3}H]MDL105,519 ((E)-3-(2)-phenyl-2-carboxyethenyl)-4,6-di-chloro-1[3H]-indole-2-carboxylicacid), a known glycine site antagonist. The amount of radioactivity displaced by the compounds is

assessed by scintillation counting. Non-specific binding is accounted for in the presence of 1 mM Glycine. Affinities are calculated from the values of % inhibition of specific [^{3}H]MDL105,519 binding by the test compounds. Indole-2-carboxylic acid is used as a positive control. The assay is commercially available at MDS Pharma Services (catalog no. 232910).

CysLT$_2$ related Assays

Radioligand Binding Assay

Compounds described herein can be evaluated for their ability to bind to the human cysteinyl (CysLT$_2$) receptor in a receptor (radioligand binding) assay (MDS pharma services, worldwide, including Taiwan, catalog # 250480). Information regarding the assay and the reagents used therein are described in Heise et al. (2000) J Biol Chem. 275:30531 and Nothacker et al. (2000) Mol Pharmacol. 58:1601.

Calcium response modulation assay

The following, as described in Lötzer et al. (Arterioscler Thromb Vasc Biol - 2003 Volume: 23(8) p. 1343), can be performed in order to assess calcium response to potential cysteinyl leukotriene receptor modulator compounds in endothelial cells and macrophages. Monocytes are be purified from peripheral blood mononuclear cells by adherence or CD14 microbead adsorption and maintained at 1x10^6 cells/mL for 5 to 9 days in RPMI-1640 medium plus 20% autologous human serum. HUVECs (human umbilical vein endothelial cells) are cultured (as described in Nuszkowski et al., *J Biol Chem.* 2001; 276: 14212–14221) and used at passages 1 to 2. HUVECs or macrophages are loaded with fura 2-AM at 4 μmol/L, and changes in calcium ions in the presence or absence of test compound or vehicle is expressed as excitation ratios of 340 nm/380 nm at 510 nm. For single-cell measurements, cells may be loaded with 2 μmol/L fluo 4-AM. Responses may be recorded on a microscope (Axiovert 200M) equipped with a confocal laser scanning head (LSM510).

Assays for Assessing Antinociception Mechanism

Compounds can be tested to determine if they influence pathways involved in nociception. The results of such assays can be used to investigate the mechanism by which a test compound mediates its antinociceptive effect. In addition to the FAAH related assays, the following methods can be used to assess the mechanism by which a test compound mediates its antinociceptive effect.

Elevation of 3α,5α-THP

3α-hydroxy-5α-pregan-20-one (3α,5α-THP or allopregnanolone) is a pregnane steroid that acts as an agonist of the inhibitory $GABA_A$ receptor subtype and is known to have both anxiolytic and analgesic effects in a variety of animal systems, with supportive evidence for a similar role in humans. Thus, compounds that elevate 3α,5α-THP may have an antinociceptive effect. The level of 3α,5α-THP in the brain of animals treated with a test compound can be measured as described by VanDoren et al. (1982 *J* Neuroscience 20:200) as follows. Briefly, steroids are extracted from individual cerebral cortical hemispheres dissected in ice-cold saline after euthanasia. Cortices are frozen at -80 °C until use. Samples are digested in 0.3 N NaOH by sonication and extracted three times in 3 mL aliquots of 10% (v/v) ethyl acetate in heptane. The aliquots are combined and diluted with 4 mL of heptane. The extracts are applied to solid phase silica columns (Burdick & Jackson, Muskegon, MI), washed with pentane, and steroids of similar polarity to 3α,5α-THP are eluted off of the column by the addition of 25% (v/v) acetone in pentane. The eluant is then dried under N_2 and steroids are redissolved in 20% (v/v) isopropanol RIA buffer (0.1 M NaH_2P0_4, 0.9 M NaCl, 0.1% w/v BSA, pH 7.0). Extraction efficiency is determined in 50 µl of the redissolved extract by liquid scintillation spectroscopy and the remaining sample is used in the determination of 3α,5α-THP by radioimmunoassay. Reconstituted sample extracts (75 µl) and 3α,5α-THP standards (5-40,000 pg in 6.25% v/v ethanol, 31% v/v isopropyl alcohol in RIA buffer) are assayed in duplicate by the addition of 725 µl of RIA buffer, 100 µl of [^{3}H]3α,5α-THP (20,000 dpm), and 100 µl of anti-3α,5α-THP antibody. Total binding is determined in the absence of unlabeled 3α,5α-THP, and nonspecific binding is determined in the absence of antibody. The antibody-binding reaction is allowed to equilibrate for 120 minutes at room temperature and is terminated by cooling the mixture to 4 °C. Bound

3α,5α-THP is separated from unbound 3α,5α-THP by incubation with 300 µl of cold dextran coated charcoal (DCC; 0.04% dextran, 0.4% powdered charcoal in double-distilled H_2O) for 20 minutes. DCC is removed by centrifugation at 2000 x g for 10 minutes. Bound radioactivity in the supernatant is determined by liquid scintillation spectroscopy. Sample values are compared to a concurrently run 3α,5α-THP standard curve and corrected for extraction efficiency.

Cannabinoid Receptor Binding and functional activity assays

Compounds may exert an antinociceptive effect via binding to either or both of the cannabinoid receptors CB_1 and CB_2 which are G-protein coupled receptors (GPCRs) that bind the endogenous endocannabinoids, anandamide (AEA) and 2-arachidonyl glycerol (2-AG) and modulate a variety of physiological responses such as body temperature, pain, blood pressure, and intestinal motility. SR 141716A (Rimonabant) is a selective CB1 antagonist and is the being developed for the treatment of obesity. CB_1 is expressed in the brain (Matsuda et al. 1990 *Nature* 346:561), and CB_2 is expressed by macrophages and in the spleen (Munro et al. 1993 *Nature* 365:61). Both of these receptors have been implicated in mediating analgesic effects through binding of agonists (see, for example, Clayton et al. 2002 *Pain* 96:253). Thus, test compounds can be assayed to determine whether they bind to one or both human cannabinoid receptors. Cannabinoid receptor activity can be assessed in a number of ways including binding or functional assays. Examples of such assays are outlined below. An assay for CB_1 binding is described by Matsuda et al. (*supra*). This assay employs recombinant cells expressing CB_1. Binding to CB_2 can be determined in the same manner using recombinant cells expressing CB_2. Briefly, to measure the ability of a test compound to bind to CB_1, the binding of a labelled CB_1 ligand, e.g., [^{3}H]WIN 55212-2 (2 nM for CB_1 and 0.8 nM for CB_2) to membranes isolated from HEK-293 cells expressing recombinant CB_1 is measured in the presence and absence of a test compound. Non-specific binding is separately determined in the presence of several-fold excess of unlabelled WIN 55212-2 (5 µM for CB_1 and 10 µM for CB_2). The specific ligand binding to the receptors is defined as the difference between the total binding and the non-specific binding determined in the presence of an excess of unlabelled WIN 55212-2. The IC_{50} values and

Hill coefficients (n_H) are determined by non-linear regression analysis of the competition curves using Hill equation curve fitting. The inhibition constants (K_i) are calculated from the Cheng Prusoff equation ($K_i = IC_{50}/(1+(L/K_D))$, where L = concentration of radioligand in the assay, and K_D = affinity of the radioligand for the receptor).

Compounds can be evaluated for their ability to bind to the human CB1 and CB2 receptors in a radioligand binding assay such as that provided by MDS Pharma Services (worldwide, including Taiwan, catalog nos. 217020 and 217100). These assays are similar to those described by Rinaldi-Carmona et al (J Pharmacol Exp Ther (2004) 310:905-14) and Bouaboula et al, (J Biol Chem, (1995) 270:13973-80).

A binding assay is described as follows. Human CB1 (hCB1) and CB2 (hCB2) cDNAs are cloned into a vector optimized for expression of recombinant proteins in Chinese Hamster Ovary (CHO) cells. Plasmids are transfected into CHO cells by a precipitation method. CHO cells are trypsined 48 hours after transfection and selected at a density of 5 x 10^5 cells/dish into culture medium (minimum essential medium-glutamine medium, heat-inactivated dialyzed fetal calf serum (10%), gentamicin (20 mg/l), L-proline (40 mg/l), pyruvate sodium (0.5 mM), and anti-Pichia pastoris lysyl oxidase agent (1%)). After 10 days, surviving clones are recovered and cultivated in the same medium containing Fungizone (0.1%). Cells are used between the third and 22nd passages. Membranes are isolated from transfected CHO cells expressing either hCB1 or hCB2 by washing twice with phosphate-buffered saline (PBS), scraped into 50 mM Tris-HCl, pH 7.7 (buffer A), crushed in a Polytron for 1 minute at 7000 rpm/minutes, then centrifuged for 15 minutes at 1100g at 4°C. The supernatant is centrifuged for 1 hour at 105,000g. The pellet is resuspended in buffer A and protein concentration measured. Membranes are stored at -80°C until use. Alternatively, membranes containing CB1 or CB2 are prepared from the brain or the spleen of rats killed by decapitation. The brain (without the cerebellum) and the spleen are removed and homogenized for 30 seconds at 4°C in buffer A (50 mM Tris-HCl, pH7.4) in a Polytron for 30 seconds at 7000 rpm/minute then centrifuged for 10 minutes at 1100g. The supernatant is centrifuged for 30 minutes at 45,000g. The pellet is resuspended in buffer A and protein concentration measured. Membranes are stored at -80°C until use. Binding assays are performed by

incubating membranes (10–100 μg) at 30°C with the cannabinoid receptor agonist, [3H]-CP 55,940 (0.2 nM) in 1 ml of buffer A for 1 hour. A rapid filtration technique using Whatman GF/C filters (pretreated with 0.5% (w/v) polyethylenimine; Whatman, Clifton, NJ), and a 48-well filtration apparatus (Brandel Inc., Gaithersburg, MD) is used to harvest and rinse labeled membranes (3 times with 5 ml of cold buffer A containing 0.25% bovine serum albumin). The radioactivity bound to the filters is counted with 4 ml of biofluor liquid scintillant. Nonspecific binding is determined in the presence of unlabeled 1 μM CP 55,940. For selectivity studies, binding assays are carried out using standard protocols.

CB1/CB2 Functional assays.

Functional assays which monitor the G-protein coupled receptor or downstream cellular responses can be used to characterize potential agonist or antagonist activities of compounds of interest at the CB1 and CB2 receptors. Direct activation (or inhibition of activation) can be monitored using a GTPγS assay. Such assays have been described in the scientific literature and are commercially available for both CB1 and CB2 (MDS Pharma Services, worldwide, including Taiwan, catalog nos. 306000 and 306050). These assays are similar to those described by Gonsiorek et al (Mol Pharmacol (2000) 57:1045-50) and Breivogel et al (J Biol Chem (1998) 273:16865-73).

A GTPγS assay can be performed as follows. CHO-K1 cells are transfected with plasmids expressing either CB1 or CB2. Transfection can be achieved using a variety of means including calcium phosphate transfection and lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfected cells are harvested at 75% confluence with cell dissociation buffer according to the manufacturer's instructions (Life Technologies). Cells are collected by centrifugation and used immediately or stored at 80°C. Cell pellets are resuspended and incubated on ice for 30 minutes in homogenization buffer (10 mM Tris-HCl, 5 mM EDTA, and 3 mM EGTA, pH 7.6) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) as a protease and amidase inhibitor. Cells are then homogenized with 20 strokes at 900 rpm with a Dounce homogenizer with stirrer type RZR1 polytron homogenizer (Caframo, Wiarton, Ontario,

Canada). Intact cells and nuclei are removed by low-speed centrifugation (500g for 5 minutes at 4°C). Membranes in the supernatant are pelleted by centrifugation at 100,000g for 30 minutes at 4°C and then resuspended in gly-gly buffer (20 mM glycylglycine, 1 mM $MgCl_2$, and 250 mM sucrose, pH 7.2) and stored at 80°C. Protein determinations are performed with the Bradford method. ^{35}S GTPγS binding assays are performed by incubating cell membranes (1-7 μg/point, in triplicate) in the presence or absence of various compounds for 30 minutes at 30°C in GTP S binding buffer (20 mM HEPES, 100 mM NaCl, 5 mM MgCl2, and 0.2% (w/v) BSA (Factor V, lipid free), pH 7.4) supplemented with 1 to 5 μM GDP. The reaction is carried out in 96-well microplates in a final volume of 100 μl with 0.3 nM [^{35}S]GTP S (specific activity = 1250 Ci/mmol; NEN, Boston, MA). The reaction is terminated by rapid filtration of the membranes through the microfiltration plates coated with 0.5% polyethylenimine (UniFilter GF/C filter plate; Packard, Meriden, CT) with a Tomtek 96-well cell harvester (Hamden, CT). The filters are washed 10 times at room temperature with 20 mM HEPES and 10 mM sodium pyrophosphate. Membrane-bound [^{35}S]GTP S radioactivity is measured by liquid scintillation with a TopCount NXT microplate scintillation and luminescence counter (Packard). Nonlinear regression analysis of the data can be performed with Prism 2.0b (GraphPad, San Diego, CA).

Activation of the CB1 receptor also affects cell proliferation. CB1 agonists are characterized by inhibition of cellular proliferation of a breast cancer cell line (MCF-7). This has been described by Bisogno et al (Biochem J (2000) 351:817), De Petrocellis et al (Proc Natl Acad Sci USA (1998) 95:8375-80), and Melck et al (Endocrinology (2000) p118-126). Briefly, cell proliferation assays are carried out with MCF-7 cells in 6-well dishes containing subconfluent cells (at a density of about 50,000 cells/well). Test substances are introduced 3 hours after cell seeding and then daily at each change of medium. Cells are treated with trypsin and counted by a hemocytometer 4 days after the addition of test substances. No significant decrease in cell viability (as assessed by trypan blue) is observed with up to 100 μM of the CB1 agonist, anandamide. Substances are added 3 hours after cell seeding (50,000 cells/well). After 72 hours, cells are treated with trypsin and counted by a hemocytometer. Antagonist activity can be assessed by

characterizing the ability of a test compound to inhibit the anti-proliferative effects of a known CB1 agonist such as anandamide.

MEASUREMENT OF PHARMACOKINETIC PARAMETERS

To determine the various pharmacokinetic parameters, plasma samples from animals dosed with a test compound are collected and analyzed by LC/MS. Briefly, samples are prepared by protein precipitation with methanol. The supernatents from the precipitation are collected and evaporated to dryness. The dry samples are resuspended in the initial flow conditions for the HPLC. A 10 uL sample volume is injected onto Thermo Electron Hyupersil GOLD 2.1x50 analytical column. The compounds are eluted from the column with a short gradient and detected by an Applied Biosystems Sciex (Toronto, Ontario) API 4000 mass. Concentrations are determined by relative response to an internal standard and calculated based on a standard concentration curve of the test compound. Sciex Analyst Software is used to quantify the samples based on a set of prepared standards and QCs. A concentration versus time plot is generated from the data in WinNonLin (Pharsight, Corp., Mountain View, CA) to generate PK curves and PK parameters for each compound, AUC_n (Area Under the Curve, n = length of experiment in hours), oral bioavailability (F_n) is calculated using the equation: $F = (AUC_{oral} / AUC_{IV})*(Dose_{IV}/Dose_{oral})$. C_{max} and T_{max} are determined by visual inspection of the oral concentration curve. C_{max} is the maximum concentration of the test compound circulating in the blood through the duration of the experiment reported at time, T (T_{max}). The terminal half-life, $t_{1/2}$, is calculated using at least two data points on the IV curve representing the elimination phase. Thus, the $t_{1/2}$ is calculated by inserting the slope (β) of the line generated by plotting the natural log of the test compound concentration versus time (during the elimination phase) into the equation $t_{1/2} = 0.693/\beta$. The volume of distribution (Vd) is calculated using the equation $Vd = Cls/\beta$ (Cls = systemic clearance, β = slope from $t_{1/2}$ equation). Cls are determined by dividing the absolute dose by the AUC_{IV}.

ANIMAL MODELS

Animal Models For Assessing Anti-Inflammatory Activity

Any of a variety of animal models can be used to test the compounds for their effectiveness in reducing inflammation and treating pain. Useful compounds can exhibit effectiveness in reducing inflammation or pain in one or more animal models.

Carrageenan-induced foot pad edema model

The model is described, for example, by Winter et al. (1962 *Proc Soc Exp Biol Med* 111:544) and can be used to assess effects of test compounds on analgesia and/or inflammation. Briefly, animals (e.g. rats or mice) are given an oral treatment with up to three doses of a test compound, indomethacin or celecoxib, or a control vehicle (1% methylcellulose in deionized water). Thirty minutes to an hour after the last treatment, paw edema is induced by injecting 0.1-0.15 mL of a 2 % carrageenan solution into the left hindpaw. The left hindpaw volume of each rat is measured using a plethysmometer before oral treatment and at 3 hours after the injection of carrageenan. The edema volume of each animal at each time point is expressed as the change from the volume at the time of oral treatment and the anti-inflammatory effect in treated groups is expressed as % inhibition compared to the vehicle only group 3 hours after the carrageenan injection. The significance of the difference between in edema different groups is assessed by a one-way analysis of variance (ANOVA) followed by the non-paired Dunnett *t* test. In this model, hyperalgesic response and PGE_2 production can also be measured (Zhang et al. 1997 *J Pharmacol and Exp Therap* 283:1069).

Complete Freund's adjuvant (CFA) induced arthritis model

In this model arthritis is induced in groups of eight Lewis derived male rats weighing 160 ± 10 g by injecting a well-ground suspension of killed *Mycobacterium tuberculosis* (0.3 mg in 0.1 mL of light mineral oil; Complete Freund's Adjuvant, CFA) into the subplantar region of the right hind paw on Day 1. Hind paw volumes are measured by water displacement on Days 0, 1 and 5 (right hind paw, with CFA), and on Days 0, 14 and 18

(left hind paw, without CFA); rats are weighed on Days 0 and 18. Test compounds, dissolved or suspended in 2% Tween 80, are prepared fresh daily and administered orally twice daily for 5 consecutive days (Day 1 through Day 5) beginning one hour before injection of CFA. For CFA-injected vehicle control rats, the increase in paw volume on Day 5 relative to Day 1 (Acute Phase of inflammation) is generally between 0.7 and 0.9 mL; and, that on Day 18 relative to day 14 (Delayed Phase of inflammation) is generally between 0.2 and 0.4 mL. Thus, anti-inflammatory activity in this model may be denoted by values calculated during the Acute Phase as well as the Delayed Phase. Animals are also weighed on Day 0 and Day 18; CFA-injected vehicle control animals generally gain between 40 to 60 g body weight over this time period. A 30 percent or more reduction in paw volume relative to vehicle treated controls is considered of significant anti-inflammatory activity. The mean ± SEM for each treatment group is determined and a Dunnett test is applied for comparison between vehicle and treated groups. Differences are considered significant at $P<0.05$. Polyarthritis of fore paw, tail, nose and ear can be scored visually and noted on the first day and final day, wherein positive (+) sign is for swelling response and negative (-) sign is normal. X-ray radiographies of the hindpaws can also be performed for further radiological index determination of arthritic symptoms. Hyperalgesia can also be measured in this model, allowing determination of analgesic effects of test compounds (Bertorelli et al. 1999 *Brit Journ Pharmacol* 128:1252).

Air-pouch model

This model is described by Masferrer et al. (1994 *Proc Natl Acad Sci USA* 91:3228). Briefly, male Lewis rats (175-200 g, Harlan Sprague-Dawley) are subcutaneously injected with 20 mL of sterile air into the intrascapular area of the back to create air cavities. An additional 10 mL of air is injected into the cavity every 3 days to keep the space open. Seven days after the initial air injection, 2 mL of a 1% solution of carrageenan dissolved in sterile saline is injected directly into the pouch to produce an inflammatory response. In treated and untreated animals the volume of exudate is measured and the number of leukocytes present in the exudate is determined by Wright-Giemsa staining. In addition, PGE_2 and 6-keto-$PGF_{1\alpha}$ are determined in the pouch

exudates from treated and untreated animals by specific ELISAs (Cayman Chemicals, Ann Arbor, MI).

Animal Models for Assessing Analgesic Activity

Carrageenan-induced thermal hyperalgesia in rodents

This model is described by Hargreaves et al. (1988 Pain 32:77). Briefly, inflammation is induced by subplantar injection of a 2% carrageenan suspension (0.1-0.15 mL) into the right hindpaw. Three hours later, the nociceptive threshold is evaluated using a thermal nociceptive stimulation (plantar test). A light beam (44% of the maximal intensity) is focused beneath the hindpaw and the thermal nociceptive threshold is evaluated by the paw flick reaction latency (cut-off time: 30 seconds). The baseline pain threshold is measured in the right hindpaw, prior to oral treatment with the test compound or a control. The results can be expressed as the nociceptive threshold in seconds (sec) for each hindpaw and the percentage of variation of the nociceptive threshold (mean ± SEM) for each animal from the mean value of the vehicle group. A comparison of the nociceptive threshold of the paw before and after carrageenan injection in the vehicle-treated group is performed using a Student's t test, a statistically significant difference is considered for $P< 0.05$. Statistical significance between the treated groups and the vehicle group is determined by a Dunnett's test using the residual variance after a one-way analysis of variance ($P< 0.05$) using Graphpad Software.

Phenylbenzoquinone-induced writhing model

This model is described by Siegmund et al. (1957 *Proc Soc Exp Bio Med* 95:729). Briefly, one hour after oral dosing with a test compound, morphine or vehicle, 0.02% phenylbenzoquinone (PBQ) solution (12.5 mL/kg) is injected by intraperitoneal route into the mouse. The number of stretches and writhings are recorded from the 5th to the 10th minutes after PBQ injection, and can also be counted between the 35^{th} and 40^{th} minutes and between the 60^{th} and 65^{th} minutes to provide a kinetic assessment. The results are expressed as the number of stretches and writhings (mean ± SEM) and the

percentage of variation of the nociceptive threshold calculated from the mean value of the vehicle-treated group. The statistical significance of any differences between the treated groups and the control group is determined by a Dunnett's test using the residual variance after a one-way analysis of variance ($P < 0.05$) using SigmaStat Software.

Kaolin-induced arthritis model.

This model is described by Hertz et al. (1980 *Arzneim Forsch* 30:1549) and can be used to assess effects on both analgesia and inflammation. Briefly, arthritis is induced by injection of 0.1 mL of kaolin suspension into the knee joint of the right hind leg of a rat. Test compounds are administered subcutaneously after 15 minutes and again after two hours. Reference compounds can be administered orally or subcutaneously. Gait is assessed every hour from 1.5 hours to 5.5 hours after treatment and is scored as follows: normal gait (0), mild disability (1), intermittent raising of paw (2), and elevated paw (3). Results are expressed as the mean gait score (mean ± SEM) calculated from individual values at each time point and the percentage of variation of the mean score calculated from the mean value of the vehicle-treated group at 4.5 hours and 5.5 hours after treatment. The statistical significance of differences between the treated groups and the vehicle-treated group is determined by a Dunnett's test using the residual variance after a one-way analysis of variance ($P < 0.05$) at each time point.

Peripheral Mononeuropathy Model

This model is described by Bennett et al. (1988 *Pain* 33:87) and can be used to assess anti-hyperalgesic effect of an orally administered test compound in a model of peripheral mononeuropathy. The effect of the test substance can be compared to a no treatment control or reference substance, e.g., morphine. Peripheral mononeuropathy is induced by loose ligation of the sciatic nerve in anaesthetized male Sprague Dawley rats (pentobarbital; 45 mg/kg by intraperitoneal route). Fourteen days later, the nociceptive threshold is evaluated using a mechanical nociceptive stimulation (analgesimeter paw pressure test; Ugo Basile, Italy). The test and reference compounds and the vehicle are orally administered (10 mL/kg carried 1% methylcellulose). Increasing pressure is

applied to the hindpaw of the animal until the nociceptive reaction (vocalization or paw withdrawal) is reached. The pain threshold (grams of contact pressure) is measured in ipsilateral (injured) and in contralateral (non injured) hindpaws, 60 minutes after treatment. The results are expressed as: the nociceptive threshold (mean ± SEM) in grams of contact pressure for the injured paw and for the non-injured paw (vehicle-treated group) and the percentage of variation the nociceptive threshold calculated from the mean value of the vehicle-treated group. A comparison of the nociceptive threshold between the non injured paw and the injured paw of the vehicle-treated group is performed using a Student's *t* test. The statistical significance of the difference between the treated groups and the vehicle group is determined for the injured hindpaw by a Dunnett's test using the residual variance after a one-way analysis of variance ($P<0.05$) using SigmaStat Software (SigmaStat® v. 2.0.3 (SPSS Science Software, Erkrath GmbH)).

Diabetic Neuropathy Paw Pressure Test

Complete protocol details can be found in Rakieten et al. (1963 *Cancer Chemother Rep* 29:91). Briefly, diabetes is induced by intraperitoneal injection of streptozotocin in rats. Three weeks later, the nociceptive threshold is measured using the paw pressure test to assess hyperalgesia. Test compound or controls are administered intraperitoneally 30 minutes prior to pain measurement.

Acetic Acid Writhing Test

Briefly, a test compound is administered orally one hour before intraperitoneal injection of acetic acid (0.5%, 10 mL/kg) in rats. Reduction in the number of writhes by 50 percent or more (≥50) per group of animals observed during the 5 to 11 minutes period after acetic acid administration, relative to a vehicle treated control group, indicates possible analgesic activity. This assay is based on that described in Inoue, K. et al. (1991 Arzneim. Forsch./Drug Res. 41: 235).

Formalin test

Complete protocol details can be found in Hunskaar et al. (1985 Neurosci. Meth. 14:69). Briefly, 30 minutes after intraperitoneal administration of a test compound or a control, 20 µl of a 5% formalin solution is injected by subplantar route into the right hindpaw of the rat. Hindpaw licking time is recorded during the early phase and the later phase after formalin injection.

Tail flick Test

Complete protocol details can be found in D'Amour and Smith (1941 J *Pharmacol. Exp Ther.* 72:74). Briefly, 30 minutes after intraperitoneal administration of a test compound or a control, a light beam is focused onto the tail of the rat. The nociceptive reaction latency, characterized by tail withdrawal, is recorded. The cutoff time is set to 15 seconds.

Tail Immersion Test

In this test the tail of the rat is immersed into a 50-60°C water bath. The nociceptive reaction latency, characterized by tail withdrawal, is measured (Haubrich et al. 1990 *J Pharmacol Exp Ther* 255:511 and Lichtman et al. 2004 *Pain* 109:319).

Hot plate test

Complete protocol details can be found in Eddy et al. (1950 *J Pharmacol. Exp. Ther.* 98:121). Briefly, 30 minutes after intraperitoneal administration of a test compound or a control, the mouse is placed on a metallic hot plate maintained at 52°C. The nociceptive reaction latency, characterized by a licking reflex of the forepaws or by a jumping off the hot plate is recorded. The cut-off time is set to 30 seconds.

Animal Models for Assessing Anxiolytic Activity

Compounds that modulate FAAH activity, and thus fatty acid amide levels, may also have anxiolytic activity. Animal models to assess anxiolytic activity include:

Elevated Plus Maze

The elevated plus maze consists of four maze arms that originate from a central platform, effectively forming a plus sign shape as described in van Gaalen and Steckler (2000 *Behavioural Brain Research* 115:95). The maze can be made of plexiglass and is generally elevated. Two of the maze arms are unwalled (open) and two are walled (closed). The two open arms are well lit and the two enclosed arms are dark (Crawley 2000 *What's Wrong With My Mouse?: Behavioral Phenotyping of Transgenic and Knockout Mice*. Wiley-Liss, New York). The test is premised on the naturalistic conflict between the tendency of an animal to explore a novel environment and the aversive properties of a brightly lit, open area (Pellow et al. 1985 *J. Neuroscience Methods.* 14:149).

Complete protocol details can be found in Fedorova et al. (2001 *J. Pharm. Exp. Ther.* 299: 332). Briefly, following administration of test compound or control, an animal is placed individually on the central platform, facing one of the open arms opposite to the observer. The number of open and closed arm entries, and the time spent in the different compartments of the maze by the animal (central platform, open and closed arms) is scored (as described in Gaalen et al. (*supra*)). An arm visit is recorded when an animal moves all four paws into the arm as described in Simonin et al. (1998 *EMBO J.* 17: 886). Behavior is scored by an observer and/or via a video camera over a 5-minutes test session. A greater amount of time spent or entries made by the animal in the open versus the closed arms is an indicator of anxiolytic activity.

Elevated Zero Maze

The elevated zero maze is a modification of the elevated plus maze. The elevated zero maze consists of a plexiglass apparatus in the shape of a circle (i.e., a circular runway of 46 cm diameter and 5.5 cm runway width) with two open and two wall-enclosed sectors of equal size. It is elevated up to a meter above the ground. This apparatus is similar to that described in Shepherd et al., (1994 *Psychopharmacology*, 116, 56), but scaled appropriately for mice.

Complete protocol details can be found in Kathuria et al (2003 *Nature Medicine* 9: 76). Briefly, following intraperitoneal administration of test compound or control, and

an appropriate pretreatment time, an animal is placed on one open sector in front of an enclosed sector. Time in a new sector is recorded as entry with all four paws. Behavior will be scored by an observer and/or via a video camera over a 5-minutes test session. A greater amount of time spent or entries made by the animal in the open versus the walled sector is an indicator of anxiolytic activity.

Animal Models Related to Allergic Response

Any of a variety of animal models can be used to test the compounds for their effectiveness in reducing allergic and inflammatory activity. Useful compounds can exhibit effectiveness in reducing allergic response and inflammation in one or more animal models.

Systemic Eosinophilia

The model is described, for example, by Shichijo et al. (2003 *J. Pharmacol. Exp. Ther.* 307:519-520). Briefly, seven week old male Brown Norway or Wistar rats are intravenously injected with 250-300 μg/rat of 13, 14-dihydro-15-keto-prostaglandin D_2 ($DK\text{-}PGD_2$), a CRTH2 agonist (dissolved in ethanol and PBS), or the corresponding volume of solvent. Rats are pretreated with or without intravenously injected 3-30 mg/kg Ramatroban [(+)-(3R)-3-(4-fluorobenzenesulfonamido)-1,2,3,4-tetra-hydrocarbazole-9-propionic acid], a CRTH2/thromboxane A_2 antagonist (dissolved in NaOH, pH-neutralized by HCl addition, and dosed in a 10% Cremophor solution). Peripheral blood is collected at 0, 1, 2, 3, 4 and 5 hours post-injection for blood smears. Following blood collection, animals are euthanized by complete bleeding and the femoral head and condoles are removed from the left femur. Total white blood cells are counted. Differential cell counts are performed on blood smears stained with May-Gruenwald's and Giemsa's solution based on standard morphologic and histological criteria.

Induction of Contact Hypersensitivity

In this model, induction of contact hypersensitivity (CHS) is created as described by Takeshita et al. (2004. *Int. Immunol. 16(7):947-59*). On days 0 and 1, female Balb/c

mice, 7-8 weeks of age are painted onto the shaved abdominal skin with 400µl of 0.5% fluorescein isothiocyanate (FITC) dissolved in acetone:dibutylpthalate (1:1, DBP). Six days later, mice are challenged by application of 20µl of 0.5% FITC in DBP onto both sides of the right ear. The solvent control (DBP) is applied to the left ear. Challenge-induced increases in ear thickness are measured by an engineer's micrometer at 0, 24, 48 and 72 hours post-challenge. The CHS response is determined by challenge-induced increases in ear thickness. CHS response = [(right ear thickness post challenge- left ear thickness post challenge) – (right ear thickness pre challenge – left ear thickness pre challenge)].

To determine the presence of leukocyte infiltration, ears and back skins are fixed for 30 hours in zinc fixative at room temperature and embedded in paraffin for histological and immunohistochemical evaluation. For assessment of eosinophil peroxidase activity (EPO), skin sections are homogenized in 1 ml of ice cold buffer (0.05 M Tris-HCl pH 8.0 containing 0.1% Triton X-100). The tissue samples are centrifuged at 10,000 g for 20 minutes at 4° C and supernatants are collected for measurement of EPO activity. In a 96 well microtiter plate, the substrate solution (100µl of 10mM o-phenlyenediamine in 0.05 M Tris-HCl and 4mM H_2O_2) is added to the 20-fold diluted homogenate in buffer (100µl). The reaction mixture is incubated at room temperature for 1 hour before the reaction is stopped by the addition of 100µl of 2M sulfuric acid. The microtiter plate is measured for absorbance.

Evan's Blue Test

Complete protocol details can be found in Takeshita et al. (2004. *Int. Immunol. 16(7):947-59*). Briefly, female Balb/c mice, 7 weeks of age are injected at two locations intradermally on their shaved backs with increasing concentrations of 0.1-10 µg/site of DK-PGD_2. This is followed by an intravenous injection of 0.25ml of saline containing 1.25 mg of Evan's blue dye. Four hours post-dye injection, mice are euthanized and the back skin is collected. Edema severity is assessed by measuring the density of the extravasated dye. Effects of pharmacological inhibition of the inflammatory reaction to

DK-PGD_2 will also be assessed by treatment with CRTH2 antagonists, such as Ramatroban.

Ovalabumin-Induced Airway Cell Proliferation and Inflammation

Complete protocol details can be found in Eynott et al. (2003. J. Pharmacol. Ther. 304:22-29). Briefly, Brown Norway rats are sensitized on days 1, 2, and 3 with intraperitoneal (i.p.) injections of 1mg ovalbumin (OVA) and 100mg $Al(OH)_3$ in 1mL 0.9% NaCl saline. They are then exposed to either 0.9% NaCl saline or 1% OVA aerosol every 3rd day (days 6, 9, & 12) for 30 minutes. 2 mg/kg dexamethasone is used as a positive control and is dosed i.p. once a day on days 4, 5, 6, 9, & 12. Vehicle (15% β-cyclodextrins in DMSO) and test compounds are dosed orally twice a day on days 5-12. On challenge days, all animals are treated 1 hour prior to OVA allergen exposure and, if required for twice a day treatment, ~4-8 hours after allergen exposure. Samples are collected 24 hours after the last OVA challenge. For sample collection, rats are anaesthetized by administration of 10mg/kg xylazine and 60mg/kg ketamine intraperitoneally. Once the rats were fully anesthetized, blood is collected for serum via the retro-orbital route. The rats are subsequently perfused by injecting 30mL PBS through the right ventricle of the heart after the abdominal aorta is severed. A tracheostomy is then performed and bronchoalveolar lavage fluid (BAL) is collected through five 5mL rinses using Hank's Balanced Salt Solution, which was kept on ice. Airway inflammatory cell accumulation and proliferation of cells are measured through the BAL fluid collection and subsequent cell counts. Cytospin slides are prepared and eosinophil % are determined by counting ~400 cells per slide. The test compounds are dosed at 5 mg/kg twice daily. Activity is scored based on the ability of the test compound to prevent ovalbumin-induced eosinophil induction (as determined by percentage of eosinophils in BAL fluid).

Ovalbumin-induced Airway Inflammation in Sensitised Brown Norway Rats

The assay assesses the effect of test compounds on cellular recruitment into the lung after antigen challenge in the sensitised Brown Norway rat. The model is a slightly modified

protocol based on that disclosed in Underwood et al. 2002 British Journal of Pharmacology 137: 263 -275. Briefly, male Brown Norway rats (200-225g, from Harlan) are be sensitised on days 0, 14 and 21 with ovalbumin (100µg/rat, i.p.) administered with Alum™ (20mg/rat aluminium hydroxide and 20mg/rat magnesium hydroxide, i.p.). Rats are challenged with inhaled ovalbumin (10 g/l, 30 minutes) or saline aerosol on day 28. Vehicle (5 ml/kg) or test compound (1 or 10 mg/kg, 5ml/kg) are dosed orally 16 and 1 hour(s) before and 1 and 6 hours after antigen challenge. Budesonide (3 mg/kg) is included as a positive control and dosed at the same time points. End point measurements are as follows; one hour after the challenge the rats have PenH levels monitored for 5 hours to assess late asthmatic reaction.

Cellular burden and inflammatory status are assessed. Twenty-four hours after ovalbumin challenge, rats are euthanised with an overdose of pentobarbitone i.p. A heparinised blood sample is taken via cardiac puncture and the resulting plasma kept frozen. Bronchoalveolar lavage (BAL) is carried out (2 x 3 ml RPMI media, 30 seconds each). Immediately after BAL, the left lobe is removed, perfused with RPMI to remove the blood pool of cells and 300 mg of lung is chopped and stored in RPMI / FCS (fetal calf serum) containing penicillin/streptomycin. The remaining perfused, chopped lung tissue is flash frozen and stored at -80°C. The remaining lung lobes are insufflated with formalin to a pressure of 20 mmHg, the lungs tied off and stored in formalin until required.

The 300 mg of tissue undergoes collagenase digestion and the cells are recovered (For method see Underwood et al., (1997) Br. J. Pharm., 122, 439-446). Total cell counts recovered from the airway lumen and lung tissue are quantified using a Sysmex cell counter. Differential cell counts (200 cells counted which comprise eosinophils, neutrophils, lymphomononuclear cells expressed as percentage and absolute cell counts) of cells recovered from the airway lumen and lung tissue are made by light microscopy from cytocentrifuge preparations stained with Wright-Giemsa stain. Remaining BAL samples are spun down and supernatant retained at –20°C.

Sephadex induced-Pulmonary Eosinophilia in Rodents

Male Swiss Webster mice are used in a model of Sephadex induced-Pulmonary Eosinophilia. In brief, test groups receive vehicle, test compound (10 mg/kg) or positive control, dexamethasone (0.5 mg/kg), by oral gavage, twice per day (p.o., b.i.d.) at a dosing volume of 10 ml/kg, on days –1, 0, 1 and once, 4 hours pre-sacrifice, on day 2. On day 0, test groups are each intravenously administered 3mg/kg Sephadex beads G-100-120 (Sigma) at a dosing volume of 5ml/kg or no Sephadex. On day 2, four hours post vehicle/test compound/dexmethasone administration, animals are euthanized by inhalation of CO_2 and subsequently undergo histopathologic and lavage evaluation of lungs for severity of eosinophilic infiltrate in peribronchiolar locations. Bronchoalveolar lavage fluid is collected by flushing the lung via the trachea 3 times with 1 ml aliquots of cold saline, and then the lungs are harvested by filling with formalin and allowed fixation a minimum of 1 day. White blood cell counts are prepared from lavage fluids. In addition, lavage fluids are immediately prepared for cytospin and cell differential counts performed. Cytospin slides are stained with a Wrights-Giemsa stain. Whole lung sections are stained with Hematoxylin and eosin stain for morphometry evaluation of severity of inflammatory cell infiltrate in peribronchiolar locations around Sephadex beads. Three sections (initial and 2 steps at 100 μm intervals) are prepared from each animal for analysis of area or diameter of inflammation around 5-8 Sephadex beads/mouse. Morphometric digital imaging analysis is performed to score inflammation. A similar experimental protocol can be performed using Lewis rats with the modification that animals are euthanized on day 1.

Mouse Model of Allergic Airways Disease Using the FlexiVent System

In this model, animals in groups of 10 (8-10 wk old male BALB/c mice) are used to assess allergic airway disease. Mice are quarantined for 14 days. On days 0 (the first day following the end of the 14 day quarantine) and day 7, experimental animals are immunized by intraperitoneal (i.p.) injection with a mixture of ovalbumin (OVA; 10 μg) and aluminum hydroxide (Alum; 2 mg) in sterile water. A second group of animals is

immunized with sterile water only and serves as a nonimmunized (negative) control. On days 13, 14, 15, and 16, dexamethasone (positive control), test compound or vehicle only is delivered by oral gavage (all at 10 mg/kg and a dosing volume of 10 ml/kg) twice a day. Animals are exposed to ovalbumin on days 14 and 15. Ovalbumin exposures are generated by aerosolizing 1% heat-aggregated ovalbumin (chicken egg, grade V; Sigma, St. Louis, MO), diluted with filtered air, and then delivered to the exposure chambers for 3 hours (H2000, Hazelton Systems). The total mass concentration of ovalbumin is determined by gravimetric analysis of filter samples taken every hour during exposure. The target mass concentration of ovalbumin is 4 mg/m^3. Chamber temperatures are maintained at 26 ± 2°C and lights on a 12 hour on/off cycle. Animals are given food (Teklad™ certified rodent diet (Harlan Teklad, Madison, WI)), *ad libitum* except during the 3 hour exposure period. Water is available *ad libitum* throughout the duration of the study.

On day 17, animals are anesthetized and tested for pulmonary function (response to methacholine challenge) by forced oscillation techniques (FlexiVent). Airway hyperresponsiveness (AHR) to increasing concentrations of aerosolized methacholine (MCh) is measured using a FlexiVent analyzer (SCIREQ, Montreal, Canada). Briefly, each mouse is anesthetized with Avertin (250 mg/kg; 0.02 ml/g; 1.2% (w/v) solution of 2,2,2 tribromoethanol in 0.8% tert-amyl ethanol (2 methyl, 2 butanol)) i.p. and placed on a heating pad. The neck fur is shaved and a small superficial incision made in the skin above the trachea. After the lobes of the salivary gland are separated, a small incision is made in the trachea, and the trachea is cannulated with a blunt-end 20 gauge needle hub. The cannula is secured by suture thread and the skin is pulled back and secured by cyanoacrylate adhesive. Ventilation is performed through the cannula by positive pressure maneuvers on the Flexivent apparatus. Once on the ventilator, pancuronium, (paralytic, 0.5 mg/kg) is administered i.p. Heart rate is monitored via a Grass Instruments Recorder w/Tachograph. Changes in heart rate greater than 50 bpm from baseline require supplementing the anesthesia (Avertin, ip). Additional doses of Avertin are given at a dose of 100 mg/kg and the animal's heart rate is monitored for at least 60 sec to determine if additional doses are needed. After baseline measurements of resistance and compliance, increasing doses of methacholine (Mch; 3, 6, 12, 25, 50 mg/ml nebulizer) are

delivered via aerosol and resistance and compliance are measured. Airway resistance is calculated for each concentration of methacholine and the average ± SEM is plotted for all treatment groups. Changes in pulmonary resistance (i.e., Mch dose-response curves) are assessed by repeated measures two way analysis of variance (ANOVA) with Bonferroni post-test. All other statistical comparisons are made using ANOVA with the Dunnetts multiple comparison test. A value of $p<0.05$ is considered significant.

Following AHR measurements, blood is collected and saved for further evaluation. The animals are then euthanized by injection with a lethal dose of a pentobarbital-based euthanasia solution. Bronchoalveolar lavage (BAL) cells are obtained from 7 animals per experimental or control group by inserting a catheter into the trachea and lavaging the lung 3 times with 0.8 ml of PBS (without calcium chloride and magnesium chloride). Total BAL cells are determined using a hemacytometer. BAL cells are spun onto slides by cytocentrifugation and stained with a modified Wright-Giemsa stain. Four hundred cells are counted and the percentage of specific cell types determined for each animal. The first lavage fluid sample (after centrifugation) is frozen separately for future cytokine analysis. The whole lung is snap frozen dry for future analyses.

Three animals from each group which are not subjected to BAL are used for histopathologic analysis and have their lungs instilled via the trachea with 10% buffered formalin, removed and fixed in the same solution. Generally, three specimens per treatment, each consisting of multiple axial sections of lung, are examined. All sections are stained with alcian blue-H&E. Lesions are graded on a subjective basis. Lesions are graded as minimal, mild, moderate, and marked (corresponding to severity scores of 1, 2, 3, and 4, respectively) and given a distribution designation of either focal, locally extensive, multifocal, multifocal and coalescing, or diffuse (corresponding to distribution scores of 1, 2, 3, 4 and 5, respectively). The product of the severity and distribution scores is averaged for each treatment group.

Prostaglandin D_2-induced Eosinophilic Airway Inflammation

Complete protocol details can be found in Shiraishi et al (2004. *J. Pharmacol. Ther. epub as DOI:10:1124/jpet.104.078212*). Briefly, Brown Norway rats are intravenously injected with rat interleukin-5 or PBS, one hour prior to intratracheal administration of prostanoid receptor agonists. These agonists can include the following; PGD_2, two $CRTH_2$-specific agonists, DK-PGD_2, 15R-methyl PGD_2, and 11-deoxy-11-methylene-15-keto-PGD_2 (MK-PGD_2), a DP receptor-specific agonist BW 245C, a thromboxane A_2 receptor (TP)-specific agonist, -BOP and Indomethacin. In some experiments, an orally delivered CRTH2/TP antagonist, Ramatroban, an intravenously delivered DP antagonist, BW A868C, or an intravenously delivered TP antagonist are administered two hours prior to administration of agonists. Rats are euthanized at 2, 8 and 24 hours post-agonist administration. Inflammatory cell accumulation in the trachea and lungs is recovered by bronchoalveolar lavage for cell counts and lungs are evaluated by histological examination. In a separate experiment, rats receive intravenous injection of IL-5 (0.2 ng/kg) or PBS one hour prior to intratracheal administration of PGD_2 (100 nmoles/animal) or vehicle. A peripheral blood sample is collected hourly post-dose of IL-5 for hematological evaluation.

Murine Allergic Inflammation

Complete protocol details are described in Fujitani et al. (2002 J. Immunol 168:443-449) and Matsuoka et al. (2000. *Science 287: 2013-2017*). Briefly, transgenic and wildtype mice are immunized with 10 μg ovalbumin (OVA) in 0.2 ml aluminum hydroxide (Alum) on days 0 and 14. On day 21, the mice are exposed to aerosolized OVA (50mg/ml in sterile saline) for 20 minutes. On days 1 and 3 post-OVA challenge, mice are euthanized, bronchoalveolar lavaged, and the lavage fluid is assessed by differential cell counting.

Allergic Rhinitis in Anesthetized Rodents

In this model described, for example, by Arimura et al. (2001 *J. Pharmacol. Ther. 298:411-419)* guinea pigs are sensitized to OVA twice by inhalation of an aerosol solution of 1 % OVA for 10 minutes. At 7 days after the second sensitization, the animals are anesthetized and artificially ventilated through a tracheal cannula using a respirator. Another glass cannula is inserted into the nasopharynx from the side of the

larynx, and a fixed amount of air is continuously insufflated into the nasal cavity via the nasal cannula using another respirator. Insufflation pressure is monitored by a pressure transducer connected to the side arm of the nasal cannula as an indication of intranasal pressure. Nasal antigen challenge is performed by generating an aerosol of 3% OVA between the nasal cannula and the animal respirator for 3 minutes using an ultrasonic nebulizer, and then the intranasal pressure is measured for 30 minutes. Nasal secretion and the nose are collected for further evaluation.

A biphasic allergic rhinitis model in conscious guinea pigs is also fully described in Arimura et al. (2001 *J. Pharmacol. Ther. 298:411-419).*

Allergic Conjunctivitis Model

Complete protocol details are described in Arimura et al. (2001 *J. Pharmacol. Ther. 298:411-419).* Briefly, a 2.5% OVA solution is applied topically to both eyes (10 µl/eye) of conscious guinea pigs that have been sensitized as described in the "Allergic Rhinitis Model in Anesthetized Rodents" protocol above. Immediately following OVA application, Evan's blue dye (20 mg/kg i.v.) is injected as a marker of plasma exudation. The amount of Evan's blue extravasated in the conjunctiva and eyelid for 30 minutes is quantified. Independently, histamine 0.001%, PGD_2 0.01%, or a combination of the two are applied to the eyes of nonsensitized guinea pigs, and dye exudation is determined.

Determination of Interleukin-13 Levels in Bronchial Alveolar Lavage Fluid

A commercially available ELISA kit (Biosource, Catalog # KRC0132) is used to determine the effects of compounds on the Interleukin-13 (IL-13) levels of bronchial alveolar lavage fluid (BALF) taken from rats that have undergone certain allergen induced (e.g. ovalbumin, sephadex, prostaglandin D_2) airway cell proliferation and inflammation.

After collection, BALF samples are concentrated 5-fold with Microcon YM-3 centrifugal devices (Millipore, Catalog #42404) and stored at -80°C until use. A 500 pg/mL standard stock is prepared by reconstituting the IL-13 standard provided in the kit

with the amount of standard diluent specified on the standard vial. A standard curve is then prepared by serially the standard stock down to 7.8 pg/mL. 50 µL of each point of the standard curve and 50 µL of concentrated BALF sample are added to the ELISA plate. Added to these samples is 150 µL of anti-rat IL-13 biotin conjugate. The plate is then incubated at room temperature for 2 hours. The plate is then washed 4 times with wash buffer and 100 µL of 1-x streptavidin-peroxidase is added to all wells. The samples are then incubated at room temperature for 30 minutes. Again, the plate is washed 4 times with wash buffer. 100 µL of stabilized chromogen are added to each well and the plate is incubated at room temperature for 45 minutes. To stop the reaction, 100 µL of stop solution is added and the plate is read at 450 nm. Levels of other cytokines including IL-1β, IL-4, IL-5 and the chemokine, eotaxin can be similarly assessed in BALF samples to determine the effect of test compounds on Th-2 related function.

Determination of Ovalbumin specific Immunoglobulin E in Serum

The effects of compounds on serum immunoglobulin E (IgE) levels in rodents that have undergone allergen-induced (e.g. ovalbumin) airway cell proliferation and inflammation can be measured using an assay developed with reference to Salgado et al., Allergol. et Immunopathol., 16, 2 (95-98), 1988.

Serum samples are taken from rats suffering from asthma, induced by the inhalation of ovalbumin, and stored at -80°C until use. The ELISA plate is coated with 1.25 mg/mL ovalbumin prepared in coating buffer (0.5M Carbonate-Bicarbonate, pH 9.6, Bethyl Labs, Catalog # E107) and incubated overnight at 4°C. After 18 hours, the plate is washed one time with wash buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0, Bethyl Labs, Catalog # E106). 200 µL of blocking solution (5% skim milk/PBS) is added and the plate is incubated at 4°C for 1 hour. Serum samples are diluted 1:3000 in sample diluent (Post coat solution containing 50 mM Tris, 1% BSA, pH 8.0 0.05% Tween 20, Bethyl Labs, Catalog # E104). After the one hour incubation with blocking solution, the plate is washed three times with wash solution and 100 µL of diluted sample is added to the appropriate well. Samples are then incubated at room temperature for 3 hours. Once the 3 hour incubation is complete, the plate is washed five times with wash buffer. The sheep anti-rat IgE HRP conjugate detection antibody (Bethyl Labs, Catalog #A110-117P)

is diluted 1:100 in a 1% skim milk/PBS solution. 100 µL of this solution is then added to the plate and the plate is incubated for 1 hour at 4°C. The plate is then washed another five times with wash buffer. The TMB peroxidase substrate (Bethyl Labs, Catalog # E102) is prepared by adding equal volumes of TMB peroxidase substrate with Peroxidase solution B. 100 µL of substrate is added to plate and incubated at room temperature for 15 minutes. The enzymatic reaction is stopped by adding 100 µL of 2 M sulfuric acid (Sigma Aldrich). The plate is then read at a wavelength of 450 nm.

In the case of compounds (e.g. CRTH2 modulators, CRTH2 inhibitors) useful for treating gastrointestinal disorders in which inflammation plays a role there a number of useful animal models that can be used in the testing of compounds.

TNBS Colitis in Rats:

Complete protocol details for one model can be found in Morris et al. (Gastroenterology 96(3):795-803, 1989). Briefly, to induce chronic colonic inflammation in rats, a rubber catheter is inserted rectally into the colon such that the tip is 8 cm proximal to the anus. Next, 2,4,6-Trinitrobenzenesulfonic acid (TNBS 5-30 mg) dissolved in 50% ethanol is instilled into the lumen of the colon through the rubber atheter. Rats are euthanized at various times (24 hours and 1-8 weeks) following rectal TNBS administration and the colon tissue is examined for damage, inflammation andd ulceration. Colon weight and colonic myeloperoxidase (MPO) acitivity are also assessed.

TNBS colitis in mice:

Female C57BL/6 mice are used in a model of TNBS-Induced Colitis. Briefly, test groups each receive vehicle or test compound (i.e. test compound (10 mg/kg) or positive control (dexamethasone; 0.5 mg/kg)), by oral gavage, twice per day at a dosing volume of 10 ml/kg, on days −1, 0, 1, 2 and once, 4 hours pre-sacrifice on day 3. On day −1, mice are fasted 16-20 hours prior to TNBS injection. On day 0, mice are infused with 50 µl TNBS solution (Sigma) or vehicle/ per mouse via rectal catheter into the colon and the rectum held off for approximately 4-7 minutes. Animals are returned to cages and

monitored for full recovery. Behavior is monitored daily. Body weights are measured each day and at termination. Mice are euthanized by cervical dislocation and necropsied on day 3 (72 hours post- TNBS injection) for assessment of gross observations, colon clinical observations and collection of all colons into 10% neutral buffered formalin for histopathologic evaluation. Clinical assessments include colon length, colon weight, hemorrhage, stricture formation, ulceration, fecal blood, mucus, diarrhea, erythema, adhesion and edema at necropsy. Colon histopathology quantitates the extent of inflammation (e.g. foamy macrophage, lymphocyte and polymorphonuclear cell infiltrate), gland loss and epithelial loss by clinical scoring of severity and percentage area affected. Scoring is performed in a blinded manner. Colon tissue may also be assessed in an *in vitro* myeloperoxidase (MPO) assay for MPO enzyme activity. Complete protocol details for an alternative model can be found in Dohi et al. (Gastroenterology 119:724-733, 2000). Briefly, mice (C57BL/6; 40 µg/g and Balb/c 36 µg/g) are given a solution of TNBS dissolved in a mixture of phosphate-buffered saline and then mixed with an equal volume of ethanol for a final concentration of 2% TNBS in 50% ethanol. On days 0 and 7, the TNBS enema is administered to mice anesthetized with ketamine and xylazine via a glass microsyringe equipped with a gastric intubation needle. Tissues and cells are assessed 3 days later (day 10).

Oxazolone Colitis in Mice

Complete protocol details for this model can be found in Kojima et al. (J. Pharmacol. Sci. 96:307-313, 2004). Briefly, a metal catheter is inserted 4 cm into the lumen of the colon via the anus in the anesthetized mouse. Oxazolone solution (0.15 mL/mouse) is administered into the colon through the catheter. Colonic tissues from mice on days 0 (before colitis induction), 1, 2, 4 and 7 are collected and examined for evidence of colitis and myeloperoxidase (MPO) activity.

In general any model of colitis can be used, in particular, mouse or rat models in which a chemical, hapten or antigen is used to induce colitis.

Oral antigen-induced gastrointestinal allergy in mice

Complete protocol details for one model can be found in Hogan et al. (Nat Immunol. 2(4):353-60, 2001). Briefly, mice are sensitized by intraperitoneal injection with ovalbumin (50 μg) in aluminum hydroxide (alum;1mg) in 0.9% sterile saline on day 0. On days 12 and 15, mice are orally administered with encapsulated ovalbumin or placebo enteric-coated beads (20mg) followed by oral administration of acidified water (300 μl, pH 2.0). In some experiments, mice are intragastrically challenged with soluble ovalbumin (1 mg) in PBS (200 μl) or control PBS on days 12 and 15. Mice are euthanized and parameters are measured 72 hours after the last antigen challenge. The gastrointestinal tract tissue is examined for for eosinophilic inflammation. Complete protocol details for another model can be found in Forbes et al. (Gastroenterology 127:105-118, 2004). Briefly, mice are sensitized by an intraperitoneal injection of 50 μg of ovalbumin/1mg of alum in 200 μL of 0.9% sterile saline on day 0. On days 12, 14, and 16, mice are orally administered 20 mg of either encapsulated ovalbumin enteric coated beads or placebo beads, followed by 200μL of acidified water 9 pH 2.0). 72 hours after the last antigen challenge, mice are euthanized and disease parameters are measured in various ways. In some experiments, mice are intraperitoneally injected on days 0, 1, and 3 with either rat IgG2b-depleting anti-D4 monoclonal antibody or rat IgG control antibody. Methacholine-induced bronchial hyperresponsiveness is determined on day 4.

Myeloperoxidase Assay

This protocol has been modified from descriptions in Arita et. al. (2005 *Proc Natl Acad Sci USA* 102:7671) and Morris et al. (1989 *Gastroenterology* 96:795). In brief, each colon tissue sample is assessed for levels of myeloperoxidase activity. Tissues are homogenized in potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, followed by three cycles of sonication and freeze-thawing. Particulate matter is removed by centrifugation (13,000 rpm for 20 minutes at 4°C). 10 μl of supernatant is added to to 90μl of potassium phosphate buffer (pH 6.0) containing 0.2 mg/ml *o*-dianisidine dihydrochloride (ODD) and 0.0006% hydrogen peroxide. Changes in optical density are measured at 460 nm at 25°C, at 30 second and 60 second intervals up to 30 minutes.

Experimental oral allergen-induced diarrhea

Complete protocol details can be found in Brandt et al. (J. Clin. Invest. 112(11):1666-1677, 2003). Briefly, mice are sensitized twice, 2 weeks apart, with 50 μg of ovalbumin/1mg of aluminum potassium sulfate adjuvant by intraperitoneal injection. Two weeks later, mice are held in the supine position 3 times a week and orally administered 250 μL of sterile saline that contains up to 50mg of ovalbumin. Before each intragastric challenge, mice are deprived of food for 3-4 hours with the aim of limiting antigen degraduation in the stomach. Diarrhea is assessed by visually monitroing mice for up to 1 hour following intragastric challenge.

Other colitis models are described Elson et al. (Gastroenterology 109:1344-1367, 1995) and Kim et al. (Scand. J. Gastroenterol 27:529-537, 1992).

Chitinase Assay

Acidic mammalian chitinase (AMCase) is induced in animal models of asthma and is found to be elevated in human asthmatics (Zhu et al, Science (2004) 304:1678-82). Therefore, AMCase may be a useful biomarker for disease and compounds which treat the causes and/or symptoms of asthma may block AMCase elevation. Direct inhibition of AMCase may also be beneficial as treatment with antibodies to AMCase have been reported to ameliorate inflammation and airway hyperresponsiveness in animal models.

Assays have been described in the scientific literature including Guo et al (J Biol Chem (2000) 275:8032-7) and Zhu et al (Science (2004) 304:1678-82). In models of asthma, mice or rats are sensitized to allergen (e.g., ovalbumin) and subsequently challenged by aerosolized antigen to induce pulmonary infiltration and airway hyper-responsiveness. Chitinase activity in bronchial alveolar lavage fluid (BALF) is assayed with the fluorogenic substrate 4-methylumbelliferyl (4-MU) (Sigma). BALF is incubated with a substrate in citrate/phosphate buffer (0.1 M/0.2 M), pH 5.2, at a concentration of 0.02 mM. After incubation at 37 °C for 15 minutes, the reaction (final volume, 110 μl) is stopped with 1 ml of 0.3 M glycine/NaOH buffer, pH 10.6, and the fluorescent 4-

methylumbelliferone released is measured with a fluorimeter (excitation, 350 nm; emission, 450 nm). A 4-methylumbelliferone (Sigma) standard curve is used to quantify the enzyme activity. Protein concentrations are determined using the Pierce micro-BCA protein assay kit. Compounds useful for the treatment of asthma may, when administered to animals at the appropriate times during the course of the experiment, reduce AMCase activity in BALF. Alternatively, compounds that directly inhibit AMCase activity when administered to animals or in an in vitro setting with purified enzyme, may also be useful for the treatment of asthma and/or allergy.

ANIMAL MODELS OF PSYCHOSIS

Animals are housed in a temperature-controlled environment with free access to food and water. Animals are allowed to become acclimatized to their new environment and are handled during 1 week before starting the experiment (to permit habituation to the investigator). All experiments are performed in a separate, quiet, light level, temperature-controlled and sound attenuated experimental room. On the test day, food and water are withdrawn during the experiment and immediately replaced after the experiment such that no animal will is without food or water for longer than 8 hours. Behavioral evaluation is observed in one or more of the following models.

Sterotypical Behavior and Hyperactivity Induced by Psychotomimetic Drugs

Each animal is individually placed into plastic test cages and allowed to habituate to the cage for up to 30 minutes prior to testing. Following habituation, animals are administered a psychotomimetic drug (such as MK-801, PCP, etc) and are then immediately replaced into the test box for behavioral observation. The stereotyped behavior and general motor activity are scored by an observer and/or via a video camera/activity monitor for up to 90 minutes post-injection (Hashimoto et al., 2005 Brain Res 1033:210-5). The test cages are thoroughly wiped clean with alcohol followed by a spray water rinse and dried after each session. This removes any olfactory cues that a rodent may leave on the test cage surface. In some cases, no drug treatment, baseline

locomotor activity measurements are taken up to 3 days prior to the test day in order to assess the natural motor activity of the animal.

Therefore, a typical study schedule for stereotyped behavior and hyperactivity progresses as follows: Animals are dosed with test compounds 1 hour prior to systemic injection of psychotomimetic drug and returned to their home cages. 30 minutes prior to behavioral testing, animals are placed in test cages to acclimate. Following habituation, animals are subcutaneously injected with a psychotomimetic drug, and placed back into their respective test cages. Behavior is recorded by an observer and/or video tracker for up to 90 minutes post injection. Following behavioral testing, animals are returned to their home cages. Animals are allowed a drug washout period of one week and behavior is re-evaluated in a counterbalanced fashion. At experiment end, animals are euthanized by CO_2 inhalation or pentobarbital overdose (>120 mg/kg). When brain tissue collection is necessary in order to analyze levels of neurotransmitters and immediate early genes, decapitation is performed. If blood sampling is necessary, it is done at the study end, after all behavioral observation is complete. To sample blood, animals are under terminal anesthesia by isoflurane or pentobarbital and sampling takes place at the retro-orbital sinus by sterile pipet tip or by cardiac puncture with a sterile needle.

Effects of Psychomimetics and Antipsychotics on Cognition (Prepulse Inhibition Model)

Startle reactivity is measured by startle chambers. Each chamber consists of a clear nonrestrictive plexiglass 8.2 cm diameter cylinder resting on a 12.5 x 25.5 cm platform inside a ventilated box. A high-frequency loudspeaker inside the chamber produces both a continuous background noise of 65 decibels (dB) and a range of acoustic dB stimuli. Vibrations of the Plexiglass cylinder caused by the whole-body startle response of the animal are transduced into analog signals by a transduction unit attached to the platform. The signals are saved to a computer. The PPI test session generally consists of a randomized presentation of startle trials (120 dB pulse), prepulse trials (60-90 dB prepulse immediately preceding a 120 dB pulse) and no stimulus trials. This session

usually lasts for 15-20 minutes. The acoustic stimuli are not harmful to the animals' hearing.

Therefore, a typical study schedule for PPI may progress as follows: Animals are dosed with test compounds or antipsychotic drugs (i.p. or s.c.). Immediately after this injection, animals are given a systemic injection (i.p. or s.c.) of either vehicle or psychotomimetic drug and 10 minutes later they are placed individually into startle chambers. A 65 dB background noise level is presented for a 10 minute acclimation period and then the PPI test session (consists of a presentation of startle trials (120 dB pulse), prepulse trials (60-90 dB prepulse immediately preceding a 120 dB pulse) and no stimulus trials) begins and lasts for 15 minutes. At the end of the test session, the animals are returned to their home cages. A no treatment, baseline measurement test session may occur up to 5-7 days prior to the drug treated test session. Following behavioral testing, animals are returned to their home cages. Animals are allowed a drug washout period of one week and behavior is re-evaluated in a counterbalanced fashion. Geyer et al. (2001) Psychopharmacology 157(2-3) 117-154 review the use of PPI models in the study of schizophrenia.

Forced swim model of depression

Compounds described herein can be screened for the ability to alleviate the depression induced in a rodent forced swim model. Examples of such protocols are found in Porsolt et al. 1977 Arch Int Pharmacodyn Ther. 229:327-336 and Porsolt et al. 1979 Eur J Pharmacol. 57:201-210.

In this model the animal is placed in plexiglass cylinder containing water from which there is no obvious means of escape. The animal alternates between vigorous swimming and immobility. The periods of immobility represent a state of despair in the animals. Animals dosed with known anti-depressants show a decrease in the duration of immobility. Periods of immobility are measured by an observer with a stop watch.

Tail suspension model of depression

A test for the screening of anti-depressant compounds is the tail suspension test. An example of the protocol can be found in Steru et al. 1985 Psychopharmacology 85 : 367-370.

This model, like the forced swim model, places animals in a situation that results in alternating vigorous movement and periods of immobility. In the assay, animals are suspended by their tails away from other objects and the floor. Like the forced swim test, animals treated with known anti-depressants show a decrease periods of immobility. These periods of immobility are measured by an observer with a stop watch.

Animal Models for Assessing Memory and Cognitive Ability

In human patients there are a number of tests that can be used to measure cognitive ability. Useful test include Mini-Mental State Examination (MMSE), Alzheimer's Disease Assessment Scale (ADAS), Boston Naming Test (BNT), and Token Test (TK). The test scores are generally analyzed by determining the percent increase or decrease over the test period compared to the baseline score at the beginning of the test period. These tests and others can be used to assess the effectiveness of the agents used for the treatment or prevention of cognitive impairment.

In analyzing candidate memory protective agents it can be useful to measure the effect of a test compound on the cognitive ability in an animal model. There are a wide range of such tests that can be used to assess candidate compounds.

One useful test involves the assessment of working memory/attention in mice. Briefly, the effect of a compound on spatial working memory can be characterized in aged mice (i.e. about 25 months old) and in young mice (i.e. about 3 months old). The working memory of the mice can first be compromised by pharmacological means (i.e. scopolamine-induced impairment). Working memory is the temporary storage of information (Bontempi et al. 2001 *J Pharm and Exp Therap* 299:297), and has been shown to be the primary type of memory disrupted in Alzheimer's disease, stroke and aging (Glasky et al. 1994 *Pharm, Biochem and Behavior* 47:325). Another useful test for assessing working memory measures Spontaneous Alternation behavior in mice. Spontaneous alternation is defined as the innate tendency of rodents to alternate free choices in a T-maze over a series of successive runs (Dember and Fowler 1958 *Psychological Bulletin* 55:412). This is a sequential procedure that relies on working

memory because the ability to alternate requires that the animal retain specific information, which varies from trial to trial (Bontempi et al. 2003 *Neuropsychopharmacology* Apr 2, 2003, 1-12). This test is also sensitive to varying parameters, such as delay intervals and increased number of trials, as well as pharmacological treatments affecting memory processes (Stefani and Gold, 2001 *Journal of Neuroscience* 21:609). In conducting this test, mice are first allowed to briefly explore a T-maze to become familiar with the apparatus. On the following day, a mouse is placed in a start box that is connected to the main stem of the T-maze. The elapsed time between the opening of the start box and the choice of an arm is measured (choice latency). The mouse is confined in the chosen arm for a set amount of time (e.g., 30 seconds) and then returned to the start box for the remaining consecutive trials in a testing session (Bontempi et al, 2003). Working memory performance for each mouse is assessed by the percentage of alternation over the trials in the testing session. Percentage is defined as entry in a different arm of the T-maze over successive trials.

The Delayed Non-Matching to Place (DNMTP) test is another useful animal model for testing the effect of a compound on cognitive ability. In this test, mice are trained and tested in an elevated eight-arm radial maze (Levin E. and Caldwell, DP (2006) Neurobiol Learn and Memory 86(1) 117-122) with a central start box placed in the center of a room with various pictures/objects placed around the room to serve as spatial cues. Each arm has a food pellet cup located at it far end. Food-deprived animals are habituated to the apparatus with all arms open and baited over a couple of successive daily free exploration periods prior to the test day. The exploration period ceases when all arms are visited and all food pellets are consumed (Bontempi et al 2001 (supra), 2003 (supra)). Animals are then trained to the DNMTP rule. A session consists of multiple trials that are separated by a defined interval. A trial consists of a study phase (two forced runs) and a test phase (two choice runs). In the study phase, the animal is given two consecutive forced runs in two different open arms. A forced run is when one arm of the maze opens allowing the animal to travel down to collect the food pellet and return to the central start box. After the second forced run, the test phase ensues. Two doors open simultaneously to begin the first choice run. One door reveals the first arm visited during the study phase and the

other is an adjacent unvisited arm. Once the animal makes a choice and then returns to the start box, the next pair of doors open (second choice run). The second choice run consists of the second arm visited in the study phase and an adjacent novel arm. During the choice runs, the animal is reinforced only when it enters the arm that had not been previously visited during the study phase. This is the non-matching to place rule; the rule being not to return to a previously visited arm. Once a mouse is trained to the DNMTP rule, variable delay periods between the study and test phases can be introduced. Mice are allowed to adapt to the delay paradigm over a few consecutive days prior to compound testing. Compound testing is conducted over a several consecutive days followed by a washout period with no paradigm training, followed by a vehicle injection for measurement of baseline performance. Test compound or vehicle injections are acutely administered prior to the start of each testing session. Working memory is evaluated by the comparison of performance on drug days versus baseline days. The effects of putative cognitive enhancing drugs are commonly evaluated in the delayed non-matching to position task (Crawley, What's Wrong With My Mouse? Behavioral Phenotyping of Transgenic and Knockout Mice, Wiley-Liss, New York, 2000). The DNMTP task is similar to schedule-induced operant tasks which include delayed matching and delayed non-matching to position tests in automated chambers, generally used in rats (Bontempi et al., 2001 (supra); Crawley, 2000 (supra)).

In addition to those working memory assays described above, another useful animal model to assess cognitive performance is the novel object recognition (NOR) assay (Ennaceur & Delacoer 1988, Behavioral Brain Res. 31, 47-49). Briefly, this assay assesses the ability of rodents to retain the memory of a "familiar" object by initially exposing them to the "familiar" object and then, after some period of time, exposing the rodent to both the "familiar" and a "novel" object. If the rodents recognize the "familiar object they will spend more time exploring the "novel" object more. If the memory of the "familiar" object is lost, rodents will investigate both objects equally. Test compounds are assessed for their ability to prolong the time period for which rodents can retain the memory of the familiar object (as measured by exploration of the novel).

Working memory tests such as those described above are thought to require identification and use of novel information on each trial (predominately affecting attentional processes) whereas spatial reference memory tasks require the same information to be used across trials.

The Morris Water Maze Task (D'Hooge and De Deyn (2001) Brain Res Rev 36 (1) 60-90) is a spatial navigation task in which an animal uses visual clues to swim to a hidden platform. Animals are motivated to find the fastest, most direct route to the platform in order to escape the water. The test typically consists of pre-training to a visible platform to test the animal's ability to conduct the procedural component of the task. Training for location of a hidden platform follows visible platform acquisition. Finally, a probe trial tests the animal's ability to find the spatial location that previously contained the hidden platform. Successful performance on the probe trial means that the animal spends significantly greater time in the trained quadrant versus non-trained quadrants. A deficit in learning and memory is defined as normal performance in the visible platform task but impaired performance on the hidden platform task.

Other tests, such as avoidance tasks, have been extensively used in the screening of compounds for cognitive enhancement (Crawley, 2000; Sarter et al. 1992 *Psychopharmacology* 107:461). For example, in the passive avoidance task, an animal is placed in a shuttle box containing a light and dark chamber (the dark is the natural preference of the rodent). The animal is trained to associate footshock with the properties of the natural preferred dark chamber. The next day, the animal is placed in the light chamber and latency to enter the dark chamber assesses the memory for the aversive association (Crawley, 2000). Potential drawbacks from these tests are that procedural components (the ability to acquire, store or retrieve memories) cannot be differentiated form declarative memory (remembering a specific item of information) as opposed to the Morris Water Maze task. Latency to enter the dark chamber on the first day is the only inherent control parameter in the avoidance task. It is known that the passive avoidance task can be affected by fear because an animal is negatively affected by the footshock so the test is often used to complement other learning and memory assays (Yamaguchi et al. 2001 *Jpn Journal of Pharmacology* 87:240).

Tests of cognitive ability are generally used in conjunction with tests designed to rule out artifacts that would impair the animal from performing complex tasks. For example, general effects on motor function (hyperactivity or sedation) can be measured by testing locomotor activity, including stereotypy (Crawley, 2000 (supra)). Motor coordination and balance can be assessed by assays such as the rotarod test. This test requires a mouse to continuously walk forward on a rotating cylinder to keep from falling off (Crawley, 2000 (supra)).

Effects of test compounds on neuropathic pain in a Spinal Nerve Ligation (SNL) Model

Compounds are evaluated in a manner similar to that described in US20050143443 example 30 (paragraph 224). Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) are given free access to food and water and are maintained on a 12:12 hour light/dark schedule for the entire duration of the study. The animal colony is maintained at 21°C and 60% humidity. The Spinal Nerve Ligation (SNL) model (Kim and Chung (1992) Pain 50:355-63) is used to induce chronic neuropathic pain. The animals are anesthetized with isoflurane, the left L5 transverse process is removed, and the L5 and L6 spinal nerves are tightly ligated with 6-0 silk suture. The wound is then closed with internal sutures and external staples. Wound clips are removed 10-11 days following surgery. The effect of test compounds on mechanical allodynia testing is determined. Baseline, post-injury and post-treatment values for non-noxious mechanical sensitivity are evaluated using 8 Semmes-Weinstein filaments (Stoelting, Wood Dale, Ill., USA) with varying stiffness (0.4, 0.7, 1.2, 2.0, 3.6, 5.5, 8.5, and 15 g) or von Frey hairs according to the up-down method (Chaplan et al. (1994) J Neurosci Methods 53:55-63). Animals are placed on a perforated metallic platform and allowed to acclimate to their surroundings for a minimum of 30 minutes before testing. The mean and standard error of the mean (SEM) are determined for each animal in each treatment group. Since this stimulus is normally not considered painful, significant injury-induced increases in responsiveness in this test are interpreted as a measure of mechanical allodynia. After a baseline reading, test compound is administered and readings are recorded every 2 hours up until 8 hours post compound administration. The anti-convulsant, gabapentin, is used as a positive control. Statistical analyses are conducted using Prism™ 4.01 (GraphPad, San Diego,

CA). Mechanical hypersensitivity of the injured paw is determined by comparing contralateral to ipsilateral paw values within the vehicle group. Data are analyzed using the Mann-Whitney test. Stability of vehicle group injured paw values over time is tested using the Friedman two-way analysis of variance by rank. Test compound effect is analyzed at each time point by carrying out a Kruskal-Wallis one-way analysis of variance by rank followed by a Dunn's post hoc test or Mann-Whitney signed rank test.

Effects of test compounds model of surgical pain

Compounds are evaluated in a manner similar to that described by Whiteside et al. (Br. J. Pharmacol (2004) 141:85-91). Briefly rats, under general anesthesia, undergo a surgical incision using aseptic technique in the plantar surface of the hind paw in a manner that incises the skin and plantar fascia of the paw starting 0.5 cm from the proximal edge of the heel and extending 1cm towards the toes. The plantaris muscle is elevated and incised longitudinally. Following hemostatsis with gentle pressure, the skin is opposed with two interrupted sutures. The wound site is treated with povidone-iodine and antibiotic powder, and the rats allowed to recover in their home cage. Twenty four hours following the surgery, thresholds to noxious stimuli may be assessed. The assessments used can include mechanical hyperalgesia using the paw pressure technique (Randall & Selitto, (1957) Arch. Int. Pharmacodynam., 3:409-419), tactile allodynia using von Frey hairs according to the up-down method (Chaplan et al. (1994) J Neurosci Methods 53:55-63), and hind limb weight bearing utilizing an incapacitance meter (Stoelting, CA). Following the measurements of basline responses 24 hours post surgery compounds can be administered via IP, PO, SC, and or IV routes, and there analgesic and/or anti-allodynic actions assessed at 1, 3, 5, and 24 hours post drug. Threshold response data are analyzed by analysis of variance (ANOVA) followed by suitable post hoc analysis such as Fischer PLSD tests.

Animal models assessing pollakiuria, urinary incontinence, and related disorders

The compounds can be assessed for their effect on cyclophosphamide induced cystitis in rats, guinea pigs, dogs, etc. as described in Ozawa et al., The Journal of Urology, the 162nd volume, the 2211 - 2216th page, 1999 and Boucher et al., The Journal of Urology, the 164th volume, the 203 - 208th page, 2000. Carlo Alberto Maggi et al. (Journal of the Autonomic Nervous System, the 38th volume, the 201 - 208th page, 1992) describe a model of overactive bladder function which can be used to assess compound activity.

Emesis Assay

The ability of compounds described herein to suppress nausea and vomiting can be assayed as described in Sharkey et al. (2007) European Journal of Neuroscience, 25: 2773–2782. Briefly, adult male ferrets (900–1500 g, Mustela putoris furo,Marshall Research Laboratories, NY, USA) are fasted overnight prior to experiments, but given free access to water. Vehicle (e.g. 2% dimethylsulfoxide, 1% Tween 80 in physiological saline) or test agent (e.g. FAAH inibitor compound at 1-2 mg/kg) are administered i.p. 15 min before the emetic agent, morphine 6 glucuronide (0.05 mg/kg, s.c., M6G, Lipomed, Arlesheim, Switzerland). The ferrets are observed for 1 h to count the number of emetic episodes (defined by rhythmic abdominal contractions with an open mouth and obvious retching) and vomiting (defined by retching with the expulsion of saliva and gastric juices). Activity is also measured in the observation period by counting the number of minutes in which the ferret makes voluntary movements. Data are presented as mean ± SEM for n-values of, for example, 5–8 animals per group. Data are compared using anova followed by a Bonferroni post hoc test and are considered statistically significant if $P < 0.05$.

Electrophysiological Assays

The compounds described herein can be assayed for their effects on human ether-a go-go gene related product (hERG) potassium channel activity. hERG channels are expressed in a human embryonic kidney (HEK293) cell line that lacks endogenous hERG channels. HEK293 cells are stably transfected with hERG cDNA. Stable transfectants are selected by coexpression with the G418-resistance gene incorporated into the expression plasmid. Selection pressure is maintained by including G418 in the culture medium. Cells are

cultured in Dulbecco's Modified Eagle Medium / Nutrient Mixture F-12 (D-MEM/F-12) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G sodium, 100 µg/mL streptomycin sulfate and 500 µg/mL G418 or similar. Cells are maintained in tissue culture incubators at 37°C in a humidified 95% air, 5% CO_2 atmosphere, with stocks maintained in cryogenic storage. Cells used for electrophysiology are plated in plastic culture dishes.

Test solution, positive control articles such as E-4031, (500 nm), terfenadine, (60 nm), or cisapride (100 nM) are prepared fresh daily in HEPES-buffered physiological saline (HB-PS) solution (composition in mM): NaCl, 137; KCl, 4.0; $CaCl_2$, 1.8; $MgCl_2$, 1; HEPES, 10; Glucose, 10; pH adjusted to 7.4 with NaOH or similar. All test and control solutions also contain 0.3% dimethylsulfoxide (DMSO). Thus the vehicle control solution is HB-PS + DMSO ≥ 0.3%.

Cells are transferred to the recording chamber and superfused with vehicle control solution. Micropipette solution for whole cell patch clamp recordings is composed of (mM): potassium aspartate, 130; $MgCl_2$, 5; EGTA, 5; ATP, 4; HEPES, 10; pH adjusted to 7.2 with KOH or similar. The recording is performed at a temperature of 35 ± 2 °C. Micropipettes for patch clamp recording are made from glass capillary tubing using a P-97 micropipette puller (Sutter Instruments, Novato, CA). A commercial patch clamp amplifier is used for whole cell recordings. Before digitization, current records are low-pass filtered at one-fifth of the sampling frequency.

Cells stably expressing hERG are held at -70- to -80 mV. Onset and steady state activation of hERG current due to test compound is measured using a pulse pattern with fixed amplitudes (conditioning prepulse: +20 to +40mV for 1 sec; repolarizing to -50 mV followed by repolarizing to -70mV repeated at 5 -10 s intervals. Each recording may end with a final application of a supramaximal concentration of a positive control article (e.g. E-4031, 500 nM), to assess the contribution of endogenous currents. The remaining unblocked current can then be subtracted off-line digitally from the data to determine the potency of the test substance for hERG activation.

Steady state is defined by the limiting constant rate of change with time (linear time dependence). The steady state before and after test compound application is used to calculate the percentage of current inhibited at each concentration. Percent activation at each concentration in the test group is compared with the vehicle control group using one-way ANOVA followed by Dunnett's multiple comparison test (JMP Version 5.0.1, SAS Institute, Cary, NC).

Test compound at different concentrations is applied to cells to determine effect on hERG current amplitude. The average value of at least 3 cells for each group ± standard error of the mean (SEM) is determined and compared to the effects of positive control articles.

SELECTIVITY ASSAYS

Measurement of Selectivity

The compounds described herein can be analyzed for target selectivity using the GPCRScreen™ (MDS Pharma Services; worldwide, including Taiwan) which screens the compounds for activity against 92 different human G protein coupled receptors.

Serine Hydrolase Selectivity Assays

Compounds (e.g. FAAH inhibitors, MAGL inhibitors, FAAH/MAGL dual inhibitors) can be tested to determine their ability to modulate (e.g. inhibit) the activity of other serine hydrolases. Thus compounds described herein can be assessed for their ability to modulate the activity (e.g. inhibit) of other serine hydrolases including the heart enzyme triacylglycerol hydrolase (TGH; Alam et al. 2002 Biochemistry 41:6679–6687), arylacetamide deacetylase (AAD; Trickett et al. 2001 J Biol Chem 276:39522–39532), carboxylesterase 1 (CE-1; Redinbo et al. 2003 Biochem Soc Trans 31:620-4), lipoprotein lipase (LPL; Stein and Stein 2003 Atherosclerosis 170:1–9) and the brain hydrolase KIAA1363 using the procedures described in Lichtman et al. 2004 Journal Pharmacol And Experimental Therapeutics 311:441-448. Furthermore, Leung et al. 2003 Nat

Biotechnol 21:687–691 describe a functional proteomic screen in which test compounds are evaluated for their ability to compete the labeling of serine hydrolases by an active site-directed FP-rhodamine probe. Test compounds are tested over a range of concentrations (100 pM–100 μM) against the soluble and membrane fractions of tissue proteomes (e.g. mouse, rat, or human brain, heart, and kidney), and from these data, IC_{50} values are determined for hydrolases that exhibit sensitivity to one or more of inhibitors. Test compound-sensitive hydrolases are then identified using biotinylated FP probes as described in Liu et al. 1999 Proc Natl Acad Sci USA 96:14694–14699 and avidin chromatography-mass spectrometry procedures, as described in Kidd et al. 2001 Biochemistry 40:4005–4015. Briefly, mouse tissues are Dounce-homogenized in tris buffer (50 mM Tris-HCl buffer, pH 8.0) with 320 mM sucrose and separated by high-speed centrifugation at 4°C. Sequential spins of 1100g for 5 minutes and 22,000g for 30 minutes yield the membrane fraction, which is washed and resuspended in Tris buffer. Supernatant from the second spin yields the soluble fraction. Proteome samples (1 mg/ml) are preincubated with test compounds over a concentration range of 100 pM to 100 μM for 10 minutes and then treated with fluorophosphonate (FP)-rhodamine (100 nM) (Patricelli et al. 2001 Proteomics 1:1067–1071) at room temperature for 10 minutes. Both test compounds and FP-rhodamine are added from concentrated DMSO stocks to give a final DMSO concentration of 2%. Reactions are quenched by the addition of 1 volume of 2x standard SDS-PAGE loading buffer (reducing), run on SDS-PAGE, and visualized in-gel using a Hitachi FMBio IIe flatbed fluorescence scanner (MiraBio, Alameda, CA). Labeled proteins are quantified by measuring integrated band intensities (normalized for volume). The band intensities of proteome samples treated with DMSO alone are considered 100% activity, and band intensities of proteins inhibited by test compounds are expressed as a percentage of remaining activity. Potent inhibitors (IC_{50} values < 10 nM) also are tested at 0.5 to 50 nM with proteome samples adjusted to 0.1 mg/ml. IC_{50} values are determined from dose-response curves from three trials at each inhibitor concentration using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Enzyme targets are affinity isolated and identified using biotinylated FP probes (Liu et al., 1999) and avidin chromatography-mass spectrometry procedures, as described previously (Kidd et al., 2001).

Anandamide uptake

Levels of anandamide can be modulated by changes in synthesis, degradation (e.g., through FAAH), and sequestration (e.g., uptake). Anandamide uptake has been described in the literature by a number of groups and small molecule inhibitors have been identified that appear to inhibit this process. In some cases, these inhibitors may also have additional pharmacological activities including inhibition of FAAH [Moore et al, Proc Natl Acad Sci (2005) 102:17852-7]. Anandamide uptake assays can be performed commercially at contract laboratories such as MDS Pharma Services (worldwide, including Taiwan, catalog no. 315500). This assay is similar to those described by Maccarrone et al (J Biol Chem (2000) 275:13484-92), Maccarrone et al (J Biol Chem (1998) 273:32332-9), Bisogno et al (J Biol Chem (1997) 272:3315-23), and Moore et al (Proc Natl Acad Sci (2005) 102:17852-7).

An anandamide uptake assay is described here. Briefly, the uptake of [1-14C] anandamide (52 mCi/mmol) can be studied in intact CHP100 or U937, a human neuroblastoma and human leukemic monocyte lymphoma line, respectively. CHP100 or U937 cells are resuspended in their serum-free culture media at a density of 1 × 106 cells/ml. Cell suspensions (2 ml/test) are incubated for different time intervals at 37 °C with 100 nM [1-14C] anandamide; then they are washed three times in 2 ml of culture medium containing 1% bovine serum albumin and are finally resuspended in 200 µl of phosphate-buffered saline. Membrane lipids are then extracted (Maccarrone, 1996, Eur J. Biochem, 241: 297-302), resuspended in 0.5 ml of methanol, and mixed with 3.5 ml of Sigma-Fluor liquid scintillation mixture for non-aqueous samples (Sigma), and radioactivity is measured in an LKB1214 Rackbeta scintillation counter. To discern non-protein-mediated from protein-mediated transport of anandamide into cell membranes, control experiments are carried out at 4 °C. Incubations (15 minutes) are also carried out with different concentrations of [1-14C] anandamide (in the 0-750 nM range) to determine apparent Km and Vmax of the uptake by Lineweaver-Burk analysis (in this case, the uptake at 4 °C is subtracted from that at 37 °C). Anandamide uptake is expressed as picomoles of anandamide taken up per minute/mg of protein. The effect of different test compounds on anandamide uptake is determined by adding each test

compound directly to the incubation medium at the selected concentrations. Cell viability after each treatment is checked with trypan blue. It is noteworthy that no specific binding of [^{3}H]CP55940, a potent cannabinoid, is not observed with plasma membranes of CHP100 cells, and U937 cells express hardly detectable levels of CB1 mRNA and very low levels of CB2 mRNA; thus, [$^{1-14}$C] anandamide binding to CB receptors is not likely to interfere in the uptake experiments.

PDE4

PDE4 is the major cAMP-metabolizing enzyme found in inflammatory and immune cells. Small molecule PDE4 inhibitors are being actively developed for the treatment of diseases associated with airway inflammation including asthma. PDE4 assays are performed commercially by contract research organizations such as MDS Pharma Services (worldwide, including Taiwan, catalog no. 154000). This assay is similar to that described by Thompson et al (Adv Cyclic Nucleotide Res (1979) 10:69-92], Nicholson et al (Trends Pharmacol Sci (1991) 12:19-27], and Cortijo et al (Br J Pharmacol (1993) 108:562-8].

Fatty acid amide hydrolase type 2 inhibition assay

The ability of test compounds to inhibit the human FAAH type 2 (huFAAH-2; FAAH-2) is assessed in an assay employing membranes protein prepared from Cos7 cells transiently transfected with huFAAH-2 cDNA (Catalog # TC106934, Origene, Rockville, MD). Briefly, 0.37 uCi/mL of oleoyl-[^{3}H]-ethanolamide (OEA, American Radiolabeled Chemicals) is diluted in buffer A (10 mM Tris-HCl, pH 6.5, 1 mM EDTA and 0.1 mg/ml of fatty acid free bovine albumin) with unlabeled OEA to yield 5 μM in the assay (200 μl volume final). Different concentration of test compounds are added and the reaction is started by adding 20 μg of membranes. After 10 min at 37°C, the reaction is stopped on ice by adding 10 ul of an acidic solution (0.5M KPO_4 adjusted at pH 2.1 with phosphoric acid). The [^{3}H]-ethanolamine product is separated from the OEA by differential adsorption to charcoal and count in a microplate scintillation counter.

Tubulin

During mitosis, a cell's DNA is replicated and then divided into two new cells. The process of separating the newly replicated chromosomes into the two forming cells involves spindle fibers constructed with microtubules, which themselves are formed by long chains of smaller protein subunits called tubulins. Spindle microtubules attach to replicated chromosomes and pull one copy to each side of the dividing cell. Without these microtubules, cell division is not possible. Tubulin inhibition assays are performed similar to those described in Bacher et al (Pure and Applied Chemistry (2001), 73:1459-1464) and Li and Sham (Expert Opinion on Therapeutic Patents (2002), 12:1663-1702) including the references described therein. Alternatively, tubulin inhibition can be assayed using the *in vitro tubulin* polymerization assay (catalog # CDS01-B; Cytoskeleton, Denver, Colorado). Microtubule-associated protein-rich lyophilized tubulin is assayed for the effect of test compounds on tubulin polymerization according to the manufacturer's recommended procedure. Briefly, each experimental compound (10ul of 10X stock) is incubated with GTP-supplemented tubulin supernatant (90 uL) in a 96-well plate. Incubation was done in a Molecular Devices plate reader at 37°C, and absorbance readings at 340 were recorded every minute for 1 hour.

PLA2

Phospholipase A2 deacylates membrane phospholipids to generate, among other products, arachidonic acid which is a precursor for the synthesis of eicosanoids including prostaglandins and leukotrienes. There are primarily 3 kinds of PLA2s: secretory (sPLA2), cytosolic calcium dependent (cPLA2), and calcium independent (iPLA2) PLA2. All 3 can degrade the synthetic substrate 2-Deoxy-2-thioarachidonoylphosphatidylcholine (arachidonoyl thio-PC) to release a free thiol that can be detected by DTNB (Dithionitrobenzoic acid). Therefore, selective inhibition of any one of the three kinds of PLA2 can be detected by measuring inhibition of thiol release in analogous reactions utilizing various purified or partially purified PLA2 sources. Additionally, there are a number of PLA2 assays that have been described in the scientific literature or are commercially available. For instance, PLA2 inhibition assays

can be performed at contract research organizations such as MDS Pharma Services (worldwide, including Taiwan, e.g., catalog nos. 160000 and 160100) or through the use of kits such as those supplied by Cayman Chemical (e.g., catalog no. 765021). Literature references include Huang et al (Anal Biochem (1994) 222:110-5), Dillard et al (J Med Chem (1996) 39:5119-36), Reynolds et al (Anal Biochem (1994) 25-32) and Snyder et al (J Pharmacol Exp Ther (1999) 288:1117-24).

THERAPEUTIC METHODS

The compounds described herein or pharmaceutically acceptable compositions thereof may be incorporated into compositions for coating implantable medical devices, such as prostheses, artificial valves, vascular grafts, stents and catheters. Accordingly, the present invention, in another aspect, includes a composition for coating an implantable device comprising a compound of the present invention as described generally above, and in classes and subclasses herein, and a carrier suitable for coating said implantable device. In still another aspect, the present invention includes an implantable device coated with a composition comprising a compound of the present invention as described generally above, and in classes and subclasses herein, and a carrier suitable for coating said implantable device.

Another aspect of the invention relates altering a biological activity in a biological sample or a patient, which method comprises administering to the patient, or contacting said biological sample with a compound described herein or a composition comprising said compound. The term "biological sample", as used herein, includes, without limitation, cell cultures or extracts thereof; biopsied material obtained from a mammal or extracts thereof; and blood, saliva, urine, feces, semen, tears, or other body fluids or extracts thereof.

Altering a biological activity in a biological sample with a compound described herein or a composition comprising said compound is useful for a variety of purposes that are known to one of skill in the art. Examples of such purposes include, but are not limited

to, blood transfusion, organ-transplantation, biological specimen storage, and biological assays.

FAAH related therapeutic methods

Compounds that inhibit FAAH activity are expected to be useful in the treatment and/or prevention of a number of disorders. FAAH inhibitors are expected to reduce one or more symptoms of one or more such disorders.

Compounds describe herein (e.g. FAAH inhibitors) can be used to prevent and/or treat, for example, epilepsy and epileptiform-iduced damage, exposure to excitotoxic neurotoxins, excitotoxicity, ischaemic brain damage, cerebral ischaema, traumatic injury (e.g. brain injury), depression, anxiety, sleep disorders, Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotropic lateral sclerosis, multiple sclerosis, tourette's syndrome, schizophrenia, glaucoma, pain, addiction, inflammation, allergic responses, eating disorders, low blood pressure, hypertension, respiratory problems, cancer (tumour growth), chemotherapy complications, asphyxia, attention deficit disorder, and gastrointestinal diseases, including nausea and vomiting, gastric ulcers, secretory diarrhea, paralytic ileus, inflammatory bowel disease, colon cancer and gastro-oesophageal reflux conditions.

Glaucoma and ocular disorders

The compounds disclosed herein (for example, FAAH inhibitor compounds) can be used to prevent and/or treat glaucoma and other disorders characterized by ocular hypertension.

Sleep Disorders

The compounds disclosed herein (for example, FAAH inhibitor compounds) can be used to prevent and/or treat a sleep disorder that affects the subject's ability to fall asleep and/or remain asleep, and/or results in unrefreshing sleep. The term "sleep disorder"

includes insomnia, night terrors, bruxism, somnambulism, sleep apnea, restless leg syndrome, unrefreshing sleep, seasonal affective disorder, circadian rhythm adjustment disorders, and the like.

Insomnia is typically classed into sleep onset insomnia, where a subject takes more than 30 minutes to fall asleep; and sleep maintenance insomnia, where the subject spends more than 30 minutes awake during an expected sleep period, or, for example, waking before the desired wake-up time with an inability to get back to sleep. Sleep disorders include both endogenous disorders, such as sleep apnea, and disorders related to behavioral or external environmental factors. For example, sleep disorders include a subject's difficulty in adjusting to a new circadian rhythm, for example, due to jet lag; night, extended, or irregular work shifts; and the like. A sleep disorder can also arise in a subject that has other disorders, diseases, or injuries, or in a subject being treated with other medications, where the subject as a result has difficulty falling asleep and/or remaining asleep, or experiences unrefreshing sleep. For example, the disclosed method is useful for inducing sleep in a subject having difficulty sleeping as the result of undergoing chemotherapy, or as a result of injuries, or as the result of stress or mood disorders such as depression, anxiety, and the like.

Sleep disorders include conditions recognized by one skilled in the art as sleep disorders, for example, conditions known in the art or conditions which are proposed to be sleep disorders or discovered to be sleep disorders. See, for example, Thorpy, MJ International Classification of Sleep Disorders, Revised: Diagnostic and Coding Manual. American Sleep Disorders Association; Rochester, Minnesota 1997; and JCD CM, International Classification of Diseases, Ninth Revision, Clinical Modification, National Center for Health Statistics, Hyattsville, MD.

Sleep disorders can be generally classed into dyssomnias, e.g., intrinsic, extrinsic, and circadian rhythm disorders; parasomnias, e.g., arousal, sleep-wake transition, and rapid eye movement (REM) associated disorders, and other parasomnias; disorders associated with mental, neurological, and other medical disorders; and other sleep disorders.

Intrinsic sleep disorders include, for example, psychophysiological insomnia, sleep state misperception, idiopathic insomnia, narcolepsy, recurrent hypersomnia, idiopathic hypersomnia, post-traumatic hypersomnia, obstructive sleep apnea syndrome, central sleep apnea syndrome, central alveolar hypoventilation syndrome, periodic limb movement disorder, restless legs syndrome, and the like.

Extrinsic sleep disorders include, for example, inadequate sleep hygiene, environmental sleep disorder, altitude insomnia, adjustment sleep disorder, insufficient sleep syndrome, limit-setting sleep disorder, sleep-onset association disorder, food allergy insomnia, nocturnal eating (drinking) syndrome, hypnotic-dependent sleep disorder, stimulant-dependent sleep disorder, alcohol-dependent sleep disorder, toxin-induced sleep disorder, and the like.

Circadian rhythm sleep disorders include, for example, time-zone change (jet lag) syndrome, shift work sleep disorder, irregular sleep-wake pattern, delayed sleep phase syndrome, advanced sleep phase syndrome, non 24h sleep-wake disorder, and the like.

Arousal sleep disorders include, for example, confusional arousals, sleepwalking, sleep terrors, and the like.

Sleep-wake transition disorders include, for example, rhythmic movement disorder, sleep starts, sleeptalking, nocturnal leg cramps, and the like.

REM-associated sleep disorders include, for example, nightmares, sleep paralysis, impaired sleep-related penile erections, sleep-related painful erections, REM sleep-related sinus arrest, REM sleep behavior disorders, and the like.

Other parasomnias include, for example, sleep bruxism, sleep enuresis, sleep-related abnormal swallowing syndrome, nocturnal paroxysmal dystonia, sudden unexplained nocturnal death syndrome, primary snoring, infant sleep apnea, congenital central

hypoventilation syndrome, sudden infant death syndrome, benign neonatal sleep myoclonus, and the like. A "sleep disorder" may also arise in a subject that has other medical disorders, diseases, or injuries, or in a subject being treated with other medications or medical treatments, where the subject as a result has difficulty falling asleep and/or remaining asleep, or experiences unrefreshing sleep, e.g., the subject experiences sleep deprivation. For example, some subjects have difficulty sleeping after undergoing medical treatment for other conditions, e.g., chemotherapy or surgery, or as a result of pain or other effects of physical injuries.

It is well known in the art that certain medical disorders, for example, central nervous system (CNS) disorders, e.g., mental or neurological disorders, e.g., anxiety, can have a sleep disorder component, e.g., sleep deprivation. Thus, treating a sleep disorder also includes treating a sleep disorder component of other disorders, e.g., CNS disorders. Further, treating the sleep disorder component of CNS disorders can also have the beneficial effect of ameliorating other symptoms associated with the disorder. For example, in some subjects experiencing anxiety coupled with sleep deprivation, treating the sleep deprivation component also treats the anxiety component. Thus, the present invention also includes a method of treating such medical disorders.

Sleep disorders associated with mental disorders include psychoses, mood disorders, anxiety disorders, panic disorder, addictions, and the like. Specific mental disorders include, for example, depression, obsessive compulsive disorder, affective neurosis/disorder, depressive neurosis/disorder, anxiety neurosis, dysthymic disorder, behavior disorder, mood disorder, schizophrenia, manic depression, delirium, alcoholism, and the like.

Sleep disorders associated with neurological disorders include, for example, cerebral degenerative disorders, dementia, parkinsonism, fatal familial insomnia, sleep related epilepsy, electrical status epilepticus of sleep, sleep-related headaches, and the like. Sleep disorders associated with other medical disorders include, for example, sleeping sickness, nocturnal cardiac ischemia, chronic obstructive pulmonary disease, sleep-related asthma,

sleep-related gastroesophageal reflux, peptic ulcer disease, fibrositis syndrome, and the like.

In some circumstances, sleep disorders are also associated with pain, e.g., neuropathic pain associated with restless leg syndrome; migraine; enhanced or exaggerated sensitivity to pain, such as hyperalgesia, causalgia and allodynia; acute pain; burn pain; atypical facial pain; neuropathic pain; back pain; complex regional pain syndromes I and II; arthritic pain; sports injury pain; pain related to infection, e.g., HIV, post-polio syndrome, and post-herpetic neuralgia; phantom limb pain; labor pain; cancer pain; postchemotherapy pain; post-stroke pain; post-operative pain; neuralgia; conditions associated with visceral pain including irritable bowel syndrome, migraine and angina; and the like.

Other sleep disorders include, for example, short sleeper, long sleeper, subwakefulness syndrome, fragmentary myoclonus, sleep hyperhidrosis, menstrual associated sleep disorder, pregnancy-associated sleep disorder, terrifying hypnagogic hallucinations, sleep-related neurogenic tachypnea, sleep-related laryngospasm, sleep choking syndrome, and the like.

Insomnia is typically classed into sleep onset insomnia, where a subject takes more than 30 minutes to fall asleep; and sleep maintenance insomnia, where the subject spends more than 30 minutes awake during an expected sleep period, or, for example, waking before the desired wake-up time with difficulty or an inability to get back to sleep. Some of the disclosed compounds are effective in treating sleep onset and sleep maintenance insomnias, insomnia resulting from circadian rhythm adjustment disorders, or insomnia resulting from CNS disorders. In one embodiment, a subject is treated for a circadian rhythm adjustment disorder. In another embodiment a subject is treated for insomnia resulting from a mood disorder. In other embodiments, a subject is treated for sleep apnea, somnambulism, night terrors, restless leg syndrome, sleep onset insomnia, and sleep maintenance insomnia. In other embodiments, a subject is treated for, sleep onset insomnia or sleep maintenance insomnia.

Compounds described herein can be used to for inducing, prolonging and/or enhancing sleep. This can encompass the treatment of a sleep disorder, i.e., a difficulty in achieving satisfactory sleep due to some internal or external factor, e.g. pain, stress or anxiety, misuse of stimulants or depressants, or temporary disturbance of lifestyle and it can encompass elective desires on the part of a user to achieve a particularly beneficial period of sleep. Such a desire may, for instance, arise in anticipation of important events the following day or in the near future for which a person may wish to be fully alert and refreshed.

The compounds can help achieve any of the following goals: getting to sleep, especially stage 1 sleep; staying asleep; sleeping well; waking refreshed; waking alert; faster onset to stage 1 sleep; increasing duration of sleep periods; decreasing the number and duration of awakenings; increasing total duration of sleep; increasing probability of sleeping well; reducing insomnia, especially chronic or mild-moderate insomnia; decreasing disturbances during sleeptime; and improving quality of sleep. Meeting these goals can be determined by any standard or, known subjective or objective measures, for instance the Karolinska scale, Loughborough sleep log or actimetry.

Improved sleep can assist in keeping awake; keeping alert; keeping refreshed; and performing well the next day

An effective amount of a compound described herein is the quantity which, when administered to a subject in need of treatment, results in the subject falling asleep more rapidly, results in more refreshing sleep, reduces duration or frequency of waking during a sleep period, or reduces the duration, frequency, or intensity of episodes of night terrors, bruxism, or somnambulism. The amount of the disclosed compound to be administered to a subject will depend on the particular disorder, the mode of administration, co-administered compounds, if any, and the characteristics of the subject, such as general health, other diseases, age, sex, genotype, body weight and tolerance to

drugs. The skilled artisan will be able to determine appropriate dosages depending on these and other factors.

The degree of refreshedness and quality of sleep may be determined by the "morning" log of the Loughborough sleep log with the highest degree of refreshedness or quality of sleep being represented as 1 and the lowest being represented as 5. Accordingly, the percentage increase in refreshedness or quality of sleep is measured in this context by the decrease in the mean refreshedness or quality of sleep.

The response of feeling extremely alert, very alert or alert can be determined, for instance, by the Karolinska 9-point scale.

Other measures of sleep parameters include the sleep disturbance index (SDI) and time to sleep onset (TTSO) that can both be measured by actimetry.

The compounds can be used in combination with therapies currently used for the treatment of sleep disorders, e.g., Aldesleukin (Proleukin), Amantadine (Symmetrel), Baclofen (Lioresal), Bepridil (Vascor), Carisoprodol (Soma), Clonazepam (Klonopin), Diazepam (Valium), Diphenhydramine (Sominex, Nytol), Doxylamine (Unisom), Estazolam (ProSom), Flurazepam (Dalmane), Gabapentin, Lorazepam (Ativan), Levodopa-carbidopa (Sinemet), Melatonin, Methylphenidate (Ritalin), Modanfinil (Provigil), Pemoline (Cylert), Pergolide, Pramipexole, Promethazine (Phenergan), Quazepam (Doral), Rimantadine (Flumadine), Sibutramine (Meridia), Sodium oxybate, Synthetic conjugated estrogens (Cenestin), Temazepam (Restoril), Triazolam (Halcion), Zaleplon (Sonata), and Zolpidem (Ambien).

Obesity related disorders

The compounds disclosed herein (for example, FAAH inhibitor compounds) may be used to treat obesity and/or to reduce or control body weight (or fat) or prevent and/or treat obesity or other appetite related disorders related to the excess consumption of food, ethanol and other appetizing substances. The compounds may be used to modulate lipid

metabolism, reduce body fat (e.g., via increasing fat utilization) or reduce (or suppress) appetite (e.g., via inducing satiety). Obesity is a condition in which there is an excess of body fat. In many cases, an individual is considered obese if the individual has a body mass index (BMA) greater than or equal to 30 kg/m^2 or if the individual has at least one co-morbidity and a BMI greater than or equal to 27 kg/m^2. In certain situations, a subject at risk for obesity is an otherwise healthy subject with a BMI of 25 kg/m^2 to less than 30 kg/m^2 or a subject with at least one co-morbidity with a BMI of 25 kg/m^2 to less than 27 kg/m^2.

The increased risks associated with obesity is thought to occur at a lower BMI in Asians. In some situations, obesity in an Asian refers to a condition whereby a subject with at least one obesity-induced or obesity-related co-morbidity that requires weight reduction or that would be improved by weight reduction, has a BMI greater than or equal to 25 kg/m^2. In Asians, an obese subject sometimes refers to a subject with at least one obesity-induced or obesity-related co-morbidity that requires weight reduction or that would be improved by weight reduction, with a BMI greater than or equal to 25 kg/m^2. In some situations, an Asian at risk of obesity is a subject with a BMI of greater than 23 kg/m^2 to less than 25 kg/m^2.

Obesity-induced or obesity-related co-morbidities include, but are not limited to, diabetes, noninsulin dependent diabetes mellitus type 2, impaired glucose tolerance, impaired fasting glucose, insulin resistance syndrome, dyslipidemia, hypertension, hyperuricacidemia, gout, coronary artery disease, myocardial infarction, angina pectoris, sleep apnea syndrome, Pickwickian syndrome, fatty liver, cerebral infarction, cerebral thrombosis, transient ischemic attack, orthopedic disorders, arthritis deformans, lumbodynia, emmeniopathy, and infertility. In particular, co-morbidities include: hypertension, hyperlipidemia, dyslipidemia, glucose intolerance, cardiovascular disease, sleep apnea, diabetes mellitus, and other obesity-related conditions.

Treatment (of obesity and obesity-related disorders) refers to the administration of the compounds described herein to reduce or maintain the body weight of an obese subject.

One outcome of treatment may be reducing the body weight of an obese subject relative to that subject's body weight immediately before the administration of the compounds described herein. Another outcome of treatment may be preventing body weight regain of body weight previously lost as a result of diet, exercise, or pharmacotherapy. Another outcome of treatment may be decreasing the occurrence of and/or the severity of obesity-related diseases. The treatment may suitably result in a reduction in food or calorie intake by the subject, including a reduction in total food intake, or a reduction of intake of specific components of the diet such as carbohydrates or fats; and/or the inhibition of nutrient absorption; and/or the inhibition of the reduction of metabolic rate; and in weight reduction in patients in need thereof. The treatment may also result in an alteration of metabolic rate, such as an increase in metabolic rate, rather than or in addition to an inhibition of the reduction of metabolic rate; and/or in minimization of the metabolic resistance that normally results from weight loss.

Prevention (of obesity and obesity-related disorders) refers to the administration of the compounds described herein to reduce or maintain the body weight of a subject at risk of obesity. One outcome of prevention may be reducing the body weight of a subject at risk of obesity relative to that subject's body weight immediately before the administration of the compounds described herein. Another outcome of prevention may be preventing body weight regain of body weight previously lost as a result of diet, exercise, or pharmacotherapy. Another outcome of prevention may be preventing obesity from occurring if the treatment is administered prior to the onset of obesity in a subject at risk of obesity. Another outcome of prevention may be decreasing the occurrence and/or severity of obesity-related disorders if the treatment is administered prior to the onset of obesity in a subject at risk of obesity. Moreover, if treatment is commenced in already obese subjects, such treatment may prevent the occurrence, progression or severity of obesity-related disorders, such as, but not limited to, arteriosclerosis, Type II diabetes, polycystic ovarian disease, cardiovascular diseases, osteoarthritis, dermatological disorders, hypertension, insulin resistance, hypercholesterolemia, hypertriglyceridemia, and cholelithiasis.

Obesity-related disorders are disorders that are associated with, caused by, or result from obesity. Examples of obesity-related disorders include overeating and bulimia, hypertension, diabetes, elevated plasma insulin concentrations and insulin resistance, dyslipidemias, hyperlipidemia, endometrial, breast, prostate and colon cancer, osteoarthritis, obstructive sleep apnea, cholelithiasis, gallstones, heart disease, abnormal heart rhythms and arrhythmias, myocardial infarction, congestive heart failure, coronary heart disease, sudden death, stroke, polycystic ovarian disease, craniopharyngioma, the Prader-Willi Syndrome, Frohlich's syndrome, GH-deficient subjects, normal variant short stature, Turner's syndrome, and other pathological conditions showing reduced metabolic activity or a decrease in resting energy expenditure as a percentage of total fat-free mass, e.g., children with acute lymphoblastic leukemia. The compounds described herein may be used to reduce or control body weight (or fat) or to prevent and/or treat obesity or other appetite related disorders related to the excess consumption of food, ethanol and other appetizing substances. The compounds may be used to modulate lipid metabolism, reduce body fat (e.g. via increasing fat utilization) or reduce (or suppress) appetite (e.g. via inducing satiety).

Further examples of obesity-related disorders are metabolic syndrome, also known as syndrome X, insulin resistance syndrome, sexual and reproductive dysfunction, such as infertility, hypogonadism in males and hirsutism in females, gastrointestinal motility disorders, such as obesity-related gastroesophageal reflux, respiratory disorders, such as obesity-hypoventilation syndrome (Pickwickian syndrome), cardiovascular disorders, inflammation, such as systemic inflammation of the vasculature, arteriosclerosis, hypercholesterolemia, hyperuricaemia, lower back pain, gallbladder disease, gout, and kidney cancer. The compounds described herein are also useful for reducing the risk of secondary outcomes of obesity, such as reducing the risk of left ventricular hypertrophy.

The compounds can be administered in combination with anti-obesity agents, including, but not limited to: 11β HSD-1 (11-beta hydroxy steroid dehydrogenase type 1) inhibitors, such as BVT 3498, BVT 2733, 3-(1-adamantyl)-4-ethyl-5-(ethylthio)- 4H-1,2,4-triazole,

3-(1-adamantyl)-5-(3,4,5-trimethoxyphenyl)-4-methyl-4H-1,2,4-triazole, 3-adamantanyl-4,5,6,7,8,9,10,11,12,3a-decahydro-1,2,4-triazolo[4,3-a][11]annulene, and those compounds disclosed in WO01/90091, WO01/90090, WO01/90092 and WO02/072084; 5HT (serotonin) transporter inhibitors, such as paroxetine, fluoxetine, fenfluramine, fluvoxamine, sertraline, and imipramine, and those disclosed in WO03/00663; 5HT antagonists such as those in WO03/037871, WO03/037887, and the like; 5HT1a modulators such as those disclosed in WO03/031439, and the like; 5HT-2 agonists; 5HT2c (serotonin receptor 2c) agonists, such as BVT933, DPCA37215, IK264, PNU 22394, WAY161503, R-1065, and YM 348 and those disclosed in U.S. Patent No. 3,914,250 and PCT publication Nos. WO02/36596, WO02/48124, WO02/10169, WO01/66548, WO02/44152, WO02/51844, WO02/40456, and WO02/40457; 5HT6 receptor modulators, such as those in WO03/030901, WO03/035061, WO03/039547, and the like; ACC2 (acetyl-CoA carboxylase-2) inhibitors; acyl-estrogens, such as oleoyl-estrone, disclosed in del Mar-Grasa, M. et al., Obesity Research, 9:202-9 (2001) and Japanese Patent Application No. JP 2000256190; alpha-lipoic acid (alpha-LA); anorectic bicyclic compounds such as 1426 (Aventis) and 1954 (Aventis), and the compounds disclosed in WO00/18749, WO01/32638, WO01/62746, WO01/62747, and WO03/015769; AOD9604; appetite suppresants such as those in WO03/40107;ATL-962 (Alizyme PLC);benzocaine;benzphetamine hydrochloride (Didrex);bladderwrack (focus vesiculosus);BRS3 (bombesin receptor subtype 3) agonists;bupropion;caffeine;CB 1 (cannabinoid-1 receptor) antagonist/inverse agonists, such as rimonabant (Acomplia; Sanofi Synthelabo), SR-147778 (Sanofi Synthelabo), BAY 65-2520 (Bayer), and SLV 319 (Solvay), and those disclosed in US Patent Nos. 4,973,587, 5,013,837, 5,081,122, 5,112,820, 5,292,736, 5,532,237, 5,624,941, 6,028,084, and 6,509,367 and WO96/33159, WO97/29079, WO98/31227, WO98/33765, WO98/37061, WO98/41519, WO98/43635, WO98/43636, WO99/02499, WO00/10967, WO00/10968, WO01/09120, WO01/58869, WO01/64632, WO01/64633, WO01/64634, WO01/70700, WO01/96330, WO02/076949, WO03/006007, WO03/007887, WO03/020217, WO03/026647, WO03/026648, WO03/027069, WO03/027076, WO03/027114, WO03/037332, WO03/040107, WO03/086940, WO03/084943 and US6,509,367 and EPO Application No. EP-658546;

CCK agonists;CCK-A (cholecystokinin-A) agonists, such as AR-R 15849, GI 181771, JMV-180, A-71378, A-71623 and SR146131, and those described in U.S. Pat. No. 5,739,106; chitosan; chromium; CNTF (Ciliary neurotrophic factors), such as GI-181771 (Glaxo-SmithKline), SR146131 (Sanofi Synthelabo), butabindide, PD170,292, and PD 149164 (Pfizer);CNTF derivatives, such as axokine (Regeneron), and those disclosed in PCT Application Nos. WO 94/09134, WO 98/22128, and WO 99/43813;conjugated linoleic acid; corticotropin-releasing hormone agonists; dehydroepiandrosterone;DGAT1 (diacylglycerol acyltransferase 1) inhibitors; DGAT2 (diacylglycerol acyltransferase 2) inhibitors; dicarboxylate transporter inhibitors; diethylpropion hydrochloride (Tenuate); dipeptidyl peptidase IV (DP-IV) inhibitors, such as isoleucine thiazolidide, valine pyrrolidide, NVP-DPP728, LAF237, P93/01, TSL 225, TMC-2A/2B/2C, FE 999011, P9310/K364, VIP 0177, SDZ 274-444 and the compounds disclosed in PCT publication Nos. WO02/083128, WO02/062764, WO03/000180, WO03/000181, WO03/000250, WO03/002530, WO03/002531, WO03/002553, WO03/002593, WO03/004498, WO03/004496,WO03/017936, WO03/024942, WO03/024965, WO03/033524, WO03/037327 and EP1258476; ephedra; exendin-4 (an inhibitor of glp-1); FAS (fatty acid synthase) inhibitors, such as Cerulenin and C75; fat resorption inhibitors such as those in WO03/053451, and the like; fatty acid transporter inhibitors; fiber (psyllium, plantago, guar fiber); galanin antagonists; galega (Goat's Rue, French Lilac); garcinia cambogia; germander (teucrium chamaedrys); ghrelin antagonists, such as those disclosed in PCT Application Nos. WO 01/87335, and WO 02/08250; GLP-1 (glucagon-like peptide 1) agonists (e.g. exendin-4); glp-1 (glucagon-like peptide-1); glucocorticoid antagonists; glucose transporter inhibitors; growth hormone secretagogue receptor agonists/antagonists, such as NN703, hexarelin, MK-0677, SM-130686, CP-424,391, L-692,429 and L-163,255, and such as those disclosed in U.S. Pat. No. 6,358,951, U.S. Patent Application Nos. 2002/049196 and 2002/022637, and PCT Application Nos. WO 01/56592 and WO 02/32888; growth hormone secretagogues, such as those disclosed and specifically described in U.S. Pat. No. 5,536,716; H3 (histamine H3) antagonist/inverse agonists, such as thioperamide, 3-(1H-imidazol-4-yl)propyl N-(4-pentenyl)carbamate), clobenpropit, iodophenpropit, imoproxifan, GT2394 (Gliatech), and A331440, and those disclosed in PCT publication No. WO02/15905 and O-[3-(1H-imidazol-4-

yl)propanol]carbamates (Kiec-Kononowicz, K. et al., Pharmazie, 55:349-55 (2000)), piperidine-containing histamine H3-receptor antagonists (Lazewska, D. et al., Pharmazie, 56:927-32 (2001), benzophenone derivatives and related compounds (Sasse, A. et al., Arch. Pharm.(Weinheim) 334:45-52 (2001)), substituted N-phenylcarbamates (Reidemeister, S. et al., Pharmazie, 55:83-6 (2000)), and proxifan derivatives (Sasse, A. et al., J. Med. Chem.. 43:3335-43 (2000)) and histamine H3 receptor modulators such as those disclosed in WO03/024928 and WO03/024929; interleukin-6 (IL-6) and modulators thereof, as in WO03/057237, and the like; L-carnitine; leptin derivatives, such as those disclosed in U.S. Pat. Nos. 5,552,524, 5,552,523, 5,552,522, 5,521,283, and PCT International Publication Nos. WO 96/23513, WO 96/23514, WO 96/23515, WO 96/23516, WO 96/23517, WO 96/23518, WO 96/23519, and WO 96/23520; leptin, including recombinant human leptin (PEG-OB, Hoffman La Roche) and recombinant methionyl human leptin (Amgen); lipase inhibitors, such as tetrahydrolipstatin (orlistat/Xenical®), Triton WR1339, RHC80267, lipstatin, teasaponin, and diethylumbelliferyl phosphate, FL-386, WAY-121898, Bay-N-3176, valilactone, esteracin, ebelactone A, ebelactone B, and RHC 80267, and those disclosed in PCT publication No. WO01/77094, and U.S. Patent Nos. 4,598,089,4,452,813, 5,512,565, 5,391,571, 5,602,151, 4,405,644, 4,189,438, and 4,242,453; lipid metabolism modulators such as maslinic acid, erythrodiol, ursolic acid uvaol, betulinic acid, betulin, and the like and compounds disclosed in WO03/011267; Mc3r (melanocortin 3 receptor) agonists; Mc4r (melanocortin 4 receptor) agonists, such as CHIR86036 (Chiron), ME-10142, ME-10145, and HS-131 (Melacure), and those disclosed in PCT publication Nos. WO99/64002, WO00/74679, WO01/991752, WO01/25192, WO01/52880, WO01/74844, WO01/70708, WO01/70337, WO01/91752, WO02/059095, WO02/059107, WO02/059108, WO02/059117, WO02/06276, WO02/12166, WO02/11715, WO02/12178, WO02/15909, WO02/38544, WO02/068387, WO02/068388, WO02/067869, WO02/081430, WO03/06604, WO03/007949, WO03/009847, WO03/009850, WO03/013509, and WO03/031410; Mc5r (melanocortin 5 receptor) modulators, such as those disclosed in WO97/19952, WO00/15826, WO00/15790, US 20030092041; MCH2R (melanin concentrating hormone 2R) agonist/antagonists; melanin concentrating hormone antagonists; melanin-concentrating hormone 1 receptor

(MCHR) antagonists, such as T-226296 (Takeda), SNP-7941 (Synaptic), and those disclosed WO01/21169, WO01/82925, WO01/87834, WO02/051809, WO02/06245, WO02/076929, WO02/076947, WO02/04433, WO02/51809, WO02/083134, WO02/094799, WO03/004027, WO03/13574, WO03/15769, WO03/028641, WO03/035624, WO03/033476, WO03/033480 and Japanese Patent Application Nos. JP 13226269, and JP1437059; melanocortin agonists, such as Melanotan II or those described in WO 99/64002 and WO 00/74679; Metformin (Glucophage®); mGluR5 modulators such as those disclosed in WO03/029210, WO03/047581, WO03/048137, WO03/051315, WO03/051833, WO03/053922, WO03/059904, and the like; monoamine reuptake inhibitors, such as sibutratmine (Meridia®/Reductil®) and salts thereof, and those compounds disclosed in U.S. Patent Nos. 4,746,680, 4,806,570, and 5,436,272, and U.S. Patent Publication No. 2002/0006964, and WO01/27068, and WO01/62341; NE (norepinephrine) transport inhibitors, such as GW 320659, despiramine, talsupram, and nomifensine; nomame herba; non-selective serotonin/norepinephrine transport inhibitors, such as sibutramine or fenfluramine; NPY 1 antagonists, such as BIBP3226, J-115814, BIBO 3304, LY-357897, CP-671906, GI-264879A, and those disclosed in U.S. Pat. No. 6,001,836, and PCT Patent Publication Nos. WO 96/14307, WO 01/23387, WO 99/51600, WO 01/85690, WO 01/85098, WO 01/85173, and WO 01/89528; NPY5 (neuropeptide Y Y5) antagonists, such as 152,804, GW-569180A, GW-594884A, GW-587081X, GW-548118X, FR235208, FR226928, FR240662, FR252384, 1229U91, GI-264879A, CGP71683A, LY-377897, LY-366377, PD-160170, SR- 120562A, SR-120819A, JCF-104, and H409/22 and those compounds disclosed in U.S. Patent Nos. 6,140,354, 6,191,160, 6,258,837, 6,313,298, 6,326,375, 6,329,395, 6,335,345, 6,337,332, 6,329,395, and 6,340,683, European Patent Nos. EP-01010691, and EP-01044970 and PCT Publication Nos. WO97/19682, WO97/20820, WO97/20821, WO97/20822, WO97/20823, WO98/27063, WO00/107409, WO00/185714, WO00/185730, WO00/64880, WO00/68197, WO00/69849, WO01/09120, WO01/14376, WO01/85714, WO01/85730, WO01/07409, WO01/02379, WO01/23388, WO01/23389, WO01/44201, WO01/62737, WO01/62738, WO01/09120, WO02/20488, WO02/22592, WO02/48152, WO02/49648, WO02/051806, WO02/094789, WO03/009845, WO03/014083, WO03/022849, WO03/028726 and Norman et al., J. Med. Chem. 43:4288-4312 (2000);